A lab-on-a-chip platform for studying the subcellular functional proteome of neuronal axons†

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Axons are long, slender processes extending from the cell bodies of neurons and play diverse and crucial roles in the development and function of nervous systems. Here, we describe the development of a chip device that can be used to produce large quantities of axons for proteomic and RNA analyses. On the chip surface, bundles of axons of rat hippocampal neurons in culture are guided to grow in areas distinct and distant from where their cell bodies reside. Fluorescence immunocytochemical studies have confirmed that the areas where these axons are guided to grow are occupied exclusively by axons and not by neuronal somatodendrites or astroglial cells. These axon-occupied parts are easily separated from the remainder of the chip and collected by breaking the chip along the well-positioned linear grooves made on the backside. One- and two-dimensional gel electrophoresis and Western blotting analyses reveal that the axons and whole cells differ in their protein compositions. RT-PCR analyses also indicate that the axons contain only a subset of neuronal RNAs. Furthermore, the chip device could be easily modified to address other issues concerning neuronal axons, such as the molecular composition of the axon substructure, the growth cone and shaft, the degeneration and regeneration processes associated with injured axons and the effects of extrinsic molecules, such as axon guidance cues and cell adhesion molecules, on the axon. With these diverse applications, the chip device described here will serve as a powerful platform for studying the functional proteome of neuronal axons.

Introduction

The axon is the longest process of a typical neuron. Axons serve as a communication line for action potential propagation and as a conduit for bi-directional trafficking of organelles and biomolecules. During development, the growth cones at the tips of axons guide them to move toward their target area.[1] Upon reaching the target area, a part of the axons differentiate into the presynaptic terminals, which work hand-in-hand with the post-synaptic terminals formed on the dendrites of the target neurons to mediate inter-cellular signaling.[2] The potential of injured axons to regenerate depends on the absence or presence of inhibitors as well as promoters in the extracellular surroundings and on the neurons' intrinsic regeneration capability.[3]

Studies have indicated that selective subsets of neuronal proteins and RNAs are present in the axon.[4–9] Axonal proteins are either synthesized in the somata and then transported to the axon *via* a cytoplasmic filter at the base of the axon[10] or locally translated from the mRNAs in the axon. Recent studies have indicated that local protein synthesis plays an important role in axonal turning behaviors and neuronal survival in response to guidance cues and neurotrophins, respectively.[11–13]

The biochemistry and cellular characteristics of axons when executing their various functions under normal conditions and the alterations in axons under pathological conditions have been studied extensively. Nerves of live animals have frequently been used to study the degeneration and regeneration processes of the axon.[14] In addition, several methods have been devised to isolate axons or processes from neurons of different animals.[4–7,15] Studies of isolated axons have begun to yield information about the protein and mRNA composition of the axon. The results accumulated thus far, albeit incomplete, have already indicated that the molecular landscape of the axon is distinct from that of the remaining somatodendritic compartments of the neuron. The correlation relationships between the proteins, either transported into the axon from the cell body or translated locally in the axon, and various axonal functions however remain to be elucidated.

We report here the development of a chip device that can be used to isolate the axons of central neurons (Fig. 1). On the chip surface, axons of cultured rat hippocampal neurons are directed to occupy regions distinct from where their somatodendritic compartments reside. By breaking the chip along well-positioned linear grooves made at the backside, the parts containing the axon-occupied regions could be easily separated from the remainder of the chip (Fig. 2). Biochemical and

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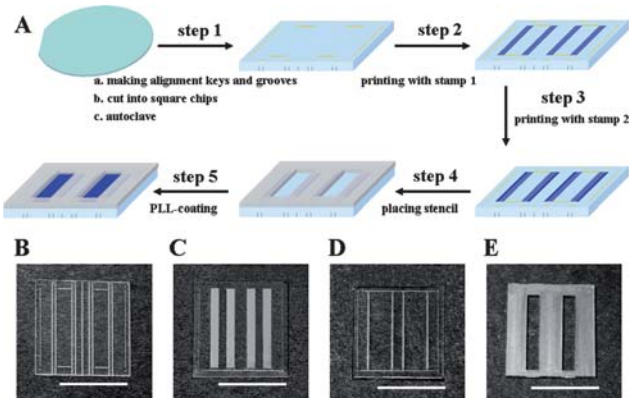

Fig. 1 Chip device fabrication. (A) The stepwise procedure used for chip device fabrication. *Step 1*: Ti/Au-coated alignment keys are made on one side of the wafer while grooves are made on the other side. The wafer is cut into 1.4 cm × 1.4 cm square chips which are then sterilized by autoclave. *Step 2*: Poly-L-lysine (PLL)-coated fine lines are printed on the glass surface by using stamp 1. *Step 3*: PLL-coated rectangular Region 2 areas are printed on glass surface by using stamp 2. *Step 4*: The PDMS stencil is placed on the glass surface. *Step 5*: The regions of glass surface not covered by stencil (the Region 1 areas) are coated with PLL. (B, C, D, E) Photographs of the glass chip, stamp 1, stamp 2 and stencil, respectively, used in the fabrication procedure. Scale bars: 1 cm.

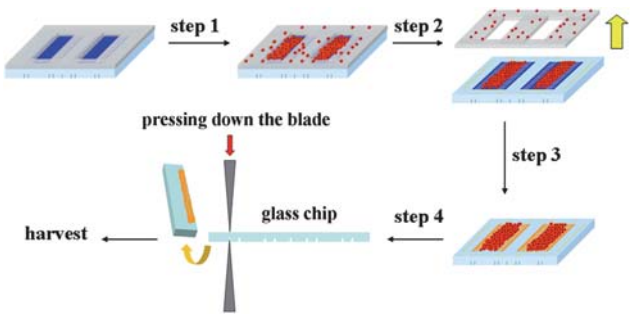

Fig. 2 Culturing neurons on and harvesting axons from the chip device. *Step 1*: Dissociated rat hippocampal neurons (red spheres) are plated into the stencil openings on top of the Region 1 areas of the glass chip. The chips are then placed in the wells of a 12-well culture plate, covered with the culture medium and kept at 37 °C in a humidified incubator under 5% CO_2/95% O_2 for 12 h. *Step 2*: The medium in the culture well is replaced with fresh medium, followed by removal of the stencil from the chip surface. *Step 3*: Neurons on the chip are further maintained in the incubator. *Step 4*: Ten to twelve days later, after axons have filled the Region 2 areas, the chip is placed between two steel blades. By pressing these blades from the top and bottom sides along the grooves cut at the backside, the chip is cleaved to generate fragments containing the various different neuronal structures. Neuronal structures on these fragments can then be harvested separately.

immunocytochemical analyses confirmed that pure axons could be harvested from the resultant chip fragments. By using this chip, and the novel chip cleaving method, pure axons in quantities sufficient for conducting systematic studies of their protein and RNA constituents are routinely produced. Furthermore, the chip device can easily be modified and used for the study of the molecular mechanisms underlying various axonal functions. The chip device described here is thus a powerful platform for exploring the functional proteome of neuronal axons.

Experimental

Chip fabrication

The procedures for fabricating the chip are described in Fig. 1A. The procedures include: step 1, making Ti/Au-coated alignment keys and cutting linear grooves on the two opposite surfaces of a glass wafer, cutting the wafer into smaller square chips (Fig. 1B) and sterilizing the chips by autoclave; step 2, printing poly-L-lysine (PLL)-coated fine lines on chip surface using stamp 1 (Fig. 1C); step 3, printing PLL-coated rectangular areas, designated as the Region 2 areas, on chip surface using stamp 2 (Fig. 1D); step 4, placing the PDMS stencil (Fig. 1E) on the chip; step 5, coating the exposed chip surface defined by the openings on the stencil, designated as the Region 1 areas, with PLL. The glass chips, stamps and stencils were prepared by conventional photolithography and soft lithography methods,[16–18] and the details of their fabrication and the materials used in this study were described in the ESI.†

Neuronal culture and axon harvesting

The procedures for growing neurons on and isolating axons from the chip are described in Fig. 2. Cells were dissociated from the hippocampi of rat fetuses at embryonic day 18 according to the procedures reported earlier.[19,20] Dissociated hippocampal neurons were first plated into the stencil openings on top of the Region 1 areas at a density of 2080 cells mm^{-2} (step 1). After 12 h, the medium on top of the stencil was replaced with fresh medium, and the stencil was lifted from the glass chip 12 h later to allow the axons to have access to the PLL-coated fine lines (step 2). The glass chips were then kept in the serum-free culture medium and maintained in a humidified incubator (step 3). The cells were treated with 5 μM cytosine-β-D-arabinofuranoside at DIV (days *in vitro*) 3 for 24 h to curtail the growth of glial cells. After 10–12 days, the chips were rinsed three times with PBS and then placed between the two steel blades of a home-made cutter (step 4, the cutter is shown in Supplementary Fig. 1 in the ESI†). By pressing these steel blades into the grooves from the top and bottom sides, a chip was cleaved into fragments that contained different neuronal structures.

Fluorescence immunocytochemistry

Fluorescence immunocytochemistry was performed according to procedures reported earlier.[21] Cultured rat hippocampal neurons on chips were fixed, permeabilized with Triton X-100-treatment and then incubated with different combinations of primary antibodies, each at the concentration of 1 μg ml^{-1} PBS, including mouse anti-βIII-tubulin antibody, rabbit anti-GFAP antibody, mouse anti-Tau antibody, rabbit anti-MAP2 antibody, mouse anti-neurofilament H antibody (SMI312), mouse anti-GAP-43 antibody and Alexa Fluor546 phalloidin. The cells were finally incubated with FITC-conjugated goat anti-mouse IgG (5 μg ml^{-1}) and Cy3-conjugated goat anti-rabbit IgG (5 μg ml^{-1}) for triple fluorescence immunostaining or with the former antibody alone at 37 °C for 1.5 h for single or double fluorescence staining. Nuclei were subsequently stained with DAPI for 15 min at room temperature. The resultant cells were examined using a confocal

microscope (LSM 510, Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RNAs were extracted from the glass fragments containing either whole cells or the axons of the cultured rat hippocampal neurons by QIAGEN RNeasy kit (QIAGEN GmbH, Hilden, Germany). Single-tube RT-PCR was performed by Titan One Tube RT-PCR system (Roche, Mannheim, Germany) with primers for each appropriate mRNA. The primer sequences (5′ to 3′) were: 18S rRNA, CCCAGTAAGTGCGGGTCATA and TTAATGATCCTTCCGCAGGT; β-actin, CCTGGGTATGGAATCCTGTG and ACATCTGCTGGAAGGTGGAC; H1 histone,[15] ACCCATTGTTCAAGGACAGC and ATCAGGTCCCCCAACTTACC; and CaMKIIα, ACGATTTCCCATCACCAGAA and CTCAGAGGATTCCTTCACGC. Reverse transcription was carried out at 50 °C for 30 min, followed by denaturing the cDNA at 94 °C for 2 min. The PCR was run for 35 cycles of amplification (10 s at 94 °C, 30 s at 55 °C, and 45 s at 68 °C), and this was followed by a 7-min extension at 68 °C.

One-and two-dimensional polyacrylamide gel electrophoresis and Western blotting

Proteins were harvested from glass fragments containing different neuronal structures by incubating in the lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% NP-40, 10mM Tris-HCl at pH 8.3, 1 mM benzamidine, 1 μg ml^{-1} pepstatin A and 1 μg ml^{-1} leupeptine) at room temperature for 5 min. One-dimensional SDS-PAGE was carried out using a mini-gel apparatus (Mini-Protean 3; Bio-Rad, Hercules, CA) and 9% polyacrylamide-gels according to the method of Laemmli.[22] Two-dimensional gel electrophoresis was performed according to the reported procedures.[23] The proteins in the polyacrylamide gels were subject to silver staining.[24] Western blotting was performed as described previously.[25] The relative intensities of the silver-stained bands were quantified by the TotalLab image analysis software system (Nonlinear Dynamics, Newcastle, UK). Bovine serum albumin with known amounts was used as the standard for protein determination.

Results and discussion

The chip (Fig. 3A) consists of a top PDMS stencil layer (1.4 cm × 1.4 cm × 1 mm) and a bottom glass chip (1.4 cm × 1.4 cm × 0.76 mm). The stencil contains two rectangular openings (2 mm × 12 mm), which are positioned over two poly-L-lysine (PLL)-coated, rectangular regions on the surface of the underlying glass chip (the Region 1 areas). Each Region 1 area is connected in turn to two other PLL-coated, rectangular regions (0.25 mm × 12 mm), the Region 2 areas, by two rows of PLL-coated fine lines (each row consisting of 218 lines of 5 μm in width and 850 μm in length, which are separated at 50 μm intervals) on the same surface. At the backside of the glass chip, nine straight wedge-shaped grooves (100 μm wide at the opening and 0.46 mm deep) have been cut by micromachining. Using the procedures described in Fig. 2, the cells dissociated from the hippocampi of rat fetuses were plated into the Region 1 areas

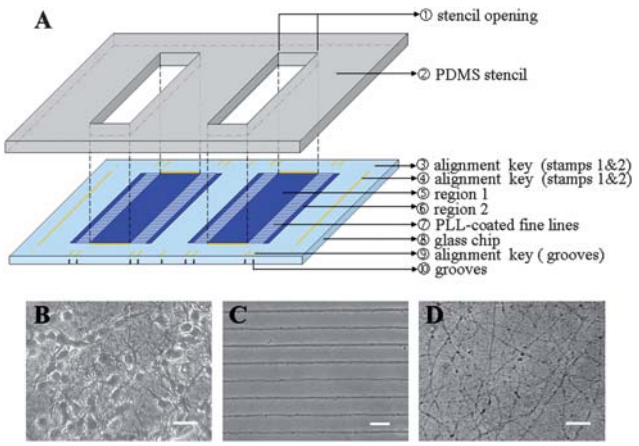

Fig. 3 Features of the chip device and neuronal structures occupying different regions of the chip surface. (A) Schematic presentation of the chip device consisting of a stencil on top of a glass chip. The structure of the stencil and the features on the surface of the glass chip are listed to the right. (B, C, D) Photomicrographs of the neuronal structures occupying the Region 1 areas, the PLL-coated fine lines and the Region 2 areas, respectively. These images were obtained from a chip device that had had dissociated rat hippocampal neurons growing on its surface for 12 days. Scale bars: 50 μm.

(Fig. 3B). As the hippocampal neurons grew, some of their axons were guided to grow along the PLL-coated fine lines (Fig. 3C) and into the Region 2 areas (Fig. 3D).

Neurons that had been grown on the chip surface for 12 days were subject to fluorescence immunocytochemical analysis using antibodies against two proteins known as markers of neuronal axons, *i.e.*, Tau[26] (Fig. 4A) and neurofilament H (SMI312)[27](Fig. 4C), and against a known marker of growth cones at the tips of growing axons, GAP-43[28] (Fig. 4D). The same neurons were also doubly immunostained with the antibody to a dendrite marker, MAP2[26] (Fig. 4A–4D). The axons, as Tau-positive processes, were found in the Region 1 areas, on PLL-coated fine lines and in the Region 2 areas (Fig. 4B-1a, -2a and -3a, respectively). These processes were also positively immunostained with anti-SMI312 and anti-GAP-43 antibodies (Fig. 4C and 4D). Growth cones (as indicated by arrows in Fig. 4*E*-1), as judged by their finger-like morphology with a peripheral F-actin-enriched domain, as indicated by positive phalloidin-labeling (Fig. 4*E*-2), and a central tubulin-positive domain[1] (Fig. 4*E*-3), were found at the tips of the axons in the Region 2 areas. The Region 1 areas also contained many dendrites, identified because the processes were positively stained by the anti-MAP2 antibody (Fig. 4B-1b). A few dendrites were found to grow along the PLL-coated fine lines (arrows in Fig. 4A), but they seldom extended beyond 200 μm from the boundary of any Region 1 area (Fig. 4B-2b). More importantly, no MAP2-positive dendrites were found in the Region 2 areas (Fig. 4A, 4C, 4D and 4B-3b).

Since rat hippocampal neuronal cultures prepared by the procedures used here also contained some astroglial cells, the distribution of neurons and astroglial cells in the different regions of the chip were also examined by triple fluorescence staining using an antibody against βIII-tubulin (a neuronal marker[29]), an antibody against GFAP (an astrocyte marker[30])

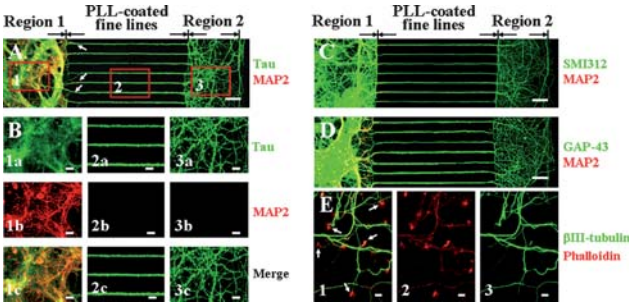

Fig. 4 Characterization of the neuronal structures within the different regions of the chip. (A) Rat hippocampal neurons on the chip surface at DIV 12 were subject to double fluorescence immunostaining with the antibodies against Tau (an axon marker, green) and against MAP2 (a dendrite marker, red). Arrows indicate MAP2-positive dendrites within the PLL-coated fine lines. (B) 1a, 2a, 3a and 1b, 2b, 3b are the areas enclosed by the red squares labeled as 1, 2 and 3 in (A) at higher magnification and immunostained with the antibodies against Tau and MAP2, respectively. 1c, 2c and 3c are the color merges of 1a and 1b, 2a and 2b, and 3a and 3b, respectively. (C) Rat hippocampal neurons on the chip surface at DIV 12 were immunostained with the antibodies against SMI312 (an axon marker, green) and against MAP2 (red). (D) Rat hippocampal neurons on the chip surface at DIV 12 were immunostained with the antibodies against GAP-43 (a growth cone marker, green) and against MAP2 (red). (E) The processes in the Region 2 areas of a chip which had had rat hippocampal neurons growing on its surface for 9 days were labeled with Alexa Fluo546 phalloidin (2, red) and immunostained with the antibody against βIII-tubulin (3, green). 1 is the color merge of 2 and 3. Arrows exemplify several growth cones. Scale bars: A, C, D, 100 μm; B, 20 μm; E, 10 μm.

and DAPI (a nucleus marker) (Fig. 5). The results indicated that almost all of the cell bodies (as indicated by DAPI-positive nuclei in Fig. 5A and 5D) and astroglial cells (cells red in color in Fig. 5A and 5C) were confined to the Region 1 areas. Although a few GFAP-positive processes and DAPI-positive nuclei were sometimes found along the PLL-coated fine lines, they seldom were present farther than 100 μm from the boundary of the Region 1 areas. No GFAP-positive cells were found in the Region 2 areas (results not shown). These observations indicated that the whole of the Region 2 areas and the majority of the fine PLL-coated lines were occupied exclusively by neuronal axons and not by the somatodendritic compartments of neurons or astroglial cells.

The linear grooves on the backside of the glass chip served as a means for facilitating the collection of the different regions of the neurons present on the chip surface. The chip could be cleaved into fragments easily and quickly by simultaneously pressing two steel blades of a home-made cutter (Supplementary Fig. 1 in the ESI†) into the grooves from the top and bottom sides. This resulted in three groups of chip fragments: those containing whole neurons plus the proximal portion of the axons growing on the PLL-coated fine lines, those containing the shafts of the axons growing on the PLL-coated fine lines and those containing the axons in the Region 2 areas (Fig. 6). Whole cells and axons could thus be harvested separately from the first and third groups of glass fragments, and the resultant samples were henceforth referred to as the whole-cell and axon samples, respectively.

Quantitative analyses indicated that each chip could generate 256.4 ± 115.0 ($n = 8$) ng of axon proteins and 8624.0 ± 532.8 ($n = 4$) ng of whole-cell proteins. When a chip was treated by the cell culture procedures as described in Fig. 2 except for no neurons being plated onto it, the chip surface was found to contain proteins at a density of 1.2 ± 0.6 ng mm^{-2} ($n = 3$). SDS-PAGE analysis of the proteins associated to the surface of cell-free chips revealed a predominant protein band with a size of 70 kDa and several minor protein bands of different sizes (left lane in Fig. 7A). This protein pattern resembled closely that of the proteins in the culture medium (data not shown). It was calculated that the chip fragments from which the axons and whole cells were collected could respectively contain up to 124 and 97 ng of proteins that were originated from the culture medium and became non-specifically associated to the chip surface during the course of cell culture. It was thus calculated that these non-specifically associated medium proteins could account for up to

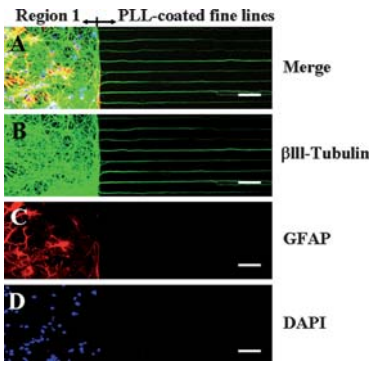

Fig. 5 Localization of neurons and astrocytes on the chip surface. Rat hippocampal neurons on the surface of a chip at DIV 12 were subject to triple fluorescence labeling using the antibody against a neuronal marker βIII-tubulin (B), the antibody against an astrocyte marker GFAP (C) and the nucleus stain DAPI (D). A is the color merge of figures B, C and D. Since no astrocytes or nuclei appear within Region 2 area, only parts of the Region 1 area and PLL-coated fine lines are shown here. Scale bars: 100 μm.

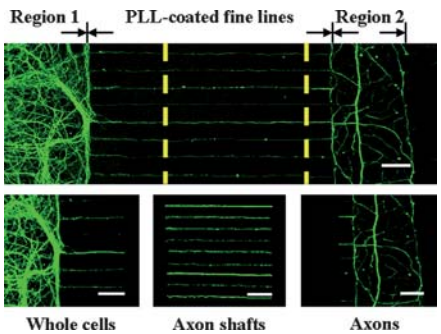

Fig. 6 Cleaving the chip into fragments containing different neuronal structures. *Upper panel*: The hippocampal neurons growing on the chip surface at DIV12 were fluorescence immunostained with the antibody against SMI312. Yellow broken lines indicate the positions of two grooves at the backside. *Lower panels*: This chip was then cleaved along these two grooves into three fragments that respectively contain whole cells and proximal axons (left), axon shafts (middle) and distal axons (right). Scale bars: 100 μm.

48% (124 ng non-specifically associated proteins/256 ng axon proteins from a single chip) and 1.1% (97 ng non-specifically associated protein/8624 ng whole-cell proteins from a single chip) of the proteins found in the harvested axon and whole-cell samples, respectively. SDS-PAGE analysis revealed striking differences between the protein patterns of the axon and whole-cell samples (Fig. 7A). The differences were clearly evident when a comparison was made between the densitometric scans of the axon proteins and whole-cell proteins after subtracting the contributions from the proteins that were non-specifically associated with the chip surface (Fig. 7B). Western blotting analysis indicated the presence of three previously known axonal proteins, Tau, GAP-43 and synaptophysin, in the axon sample, as multiple bands with sizes between 50 and 70 kDa, as two bands of 50 and 55 kDa and as a band of 38 kDa, respectively (Fig. 7C). On the other hand, the level of calcium, calmodulin-dependent protein kinase II α-subunit (CaMKIIα), a major constituent of the postsynaptic density[31] found in neuronal dendrites, in the axon sample was below the detection limit (Fig. 7C). All four of these proteins were found in the whole-cell sample. Interestingly, the immunostained pattern of Tau from the whole-cell sample (multiple bands between 50 and 70 kDa with a major band at 52 kDa) was drastically different from that in the axon sample (multiple bands between 50 and 70 kDa with two major bands at 62 and 70 kDa), indicating that the major Tau isoforms present in the axons differed from those in the somatodendritic compartments. This difference may arise from selective enrichment of certain spliced isoforms of the protein and/or the presence of certain post-translationally modified forms of the protein in the axon.[32]

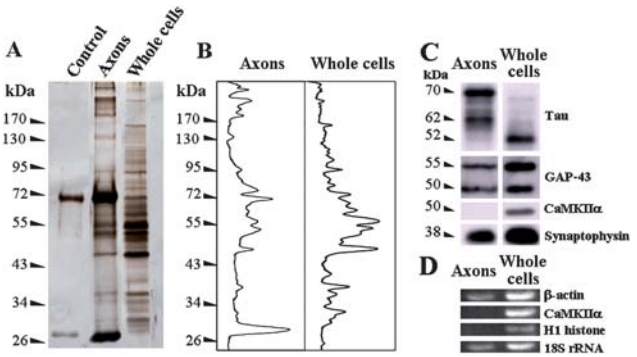

Fig. 7 Biochemical analyses of the axons isolated using the chip device. Different parts of cultured rat hippocampal neurons on the chip fragments as those shown in Fig. 6 were harvested and then subject to SDS-PAGE, Western blotting and RT-PCR analysis. (A) A silver-stained SDS-gel containing the proteins that were associated non-specifically to the chip surface (98 ng, left lane labeled as control), proteins from the axons attached to chip fragments containing the Region 2 areas (185 ng, middle lane labeled as axons) and proteins from the whole cell/proximal axons harvested attached to chip fragments containing the Region 1 areas (90 ng, right lane labeled as whole cells). Molecular weight markers are found on the left. (B) *Right and left panels*: Densitometric scans of the whole-cell proteins and the axon proteins in figure A after subtracting the scan of the control proteins. Molecular weight markers are found on the left. (C) Western blotting analyses of the axon proteins and whole-cell proteins using antibodies against Tau, CaMKIIα, GAP-43 and synaptophysin. The axon and whole-cell samples, respectively containing 277 and 130 ng protein, were used for the analysis with anti-Tau antibody. The axon and whole cell samples containing 177 and 83 ng protein, respectively, were used with the remaining antibodies. The sizes of the positively stained bands are listed on the left. (D) RT-PCR analysis of the RNAs isolated from 1/3 of the axon sample and 1/30 of the whole-cell sample harvested from a single chip by using specific primers designed for the mRNAs of β-actin, H1 Histone and CaMKIIα and 18S ribosomal RNA (18S rRNA).

The proteins in the axon and whole-cell samples were also subject to two-dimensional gel electrophoresis analysis, and the resultant gels were silver-stained (Fig. 8A and 8B). To illustrate the differences between the protein patterns of the axon and whole-cell samples, we overlaid these gels and colored the proteins isolated from axons in red and those from whole cells in green (Fig. 8C). The results indicated that these two samples shared many common proteins (as protein spots in blue color in Fig. 8C). The results also uncovered differences between these two samples as some proteins were found only in the axon (red spots in Fig. 8C, marked by arrows) while some other proteins were only found in the whole-cell sample (green spots in Fig. 8C, marked by arrow heads).

By multiplex RT-PCR, the harvested axon RNAs were found to contain β-actin mRNA and 18S ribosomal RNA, but not the mRNA for H1 histone or mRNA for CaMKIIα (Fig. 7D). These

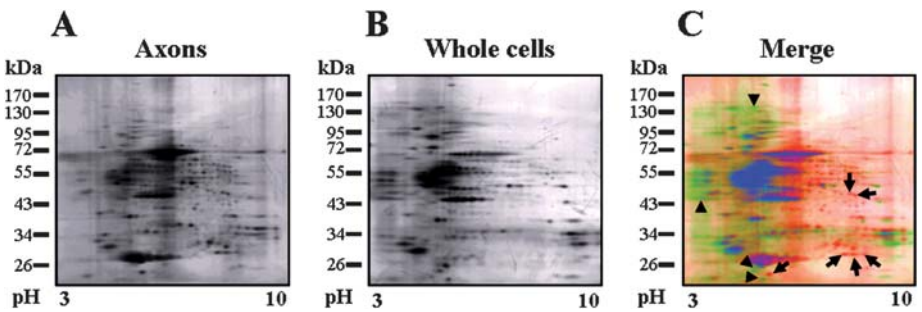

Fig. 8 Two-dimensional gel electrophoresis analyses of the proteins in the axon and whole-cell samples. 1.5 μg of axon proteins (A) and 1 μg of whole-cell proteins (B) were subjected to isoelectric focusing on non-linear pH 3–10 IGP strips. Focused proteins were then separated by 9% SDS gel and detected by silver staining. (C) Overlay of the silver-stained gel containing axon proteins (A, colored in red) and the silvered-stained gel containing the whole-cell proteins (B, colored in green). Arrows exemplify the proteins found in the axon sample and not in the whole-cell sample (protein spots in red color). Arrowheads exemplify the proteins found in the whole-cell sample and not in the axon sample (protein spots in green color). The results are from a representative experiment of a total of three experiments.

results are consistent with previous reports of the presence of β-actin mRNA[6,15,33] and the absence of CaMKIIα and H1 histone mRNAs in neuronal axons.[15] The presence of 18S ribosomal RNA, as a structural component of the small ribosomal subunit, in the axon is consistent with the notion of the presence of protein translation machinery in the axon.[11,34] The same analysis indicated that the whole-cell sample contained all of these four RNAs.

The results obtained from the above one- and two-dimensional gel electrophoresis (Fig. 7A and 7B and Fig. 8, respectively), Western blotting (Fig. 7C) and RT-PCR analyses (Fig. 7D) consistently indicate that the protein and RNA compositions of the axon are different from those of the whole cells. The results also demonstrate that the chip described in this study is a useful tool to produce pure axons in quantities large enough for carrying out the biochemical analyses that are needed to study the axon's proteome and RNA composition. Furthermore, the chip device described here may also be useful in analyzing the lipid components enriched in the axon and in detecting different signal transduction pathways in this subcellular structure by using antibodies for specific phosphorylation sites. The information shall, in the future, provide new insights into the molecular mechanisms underlying the various functions of the axon.

Several other methods have been devised to isolate axons or processes from different neurons. For example, squid giant axons[6] and the processes of *Aplysia* sensory neurons[5] have been isolated by collecting the axons (or processes) that have been physically severed from their cell bodies. Regenerating axons of dorsal root ganglion cells have been isolated using Boyden chambers.[8] For central neurons, axons have been isolated using a compartmentalized cell culture system devised by Campenot[4,35] or a microfluidic cell culture platform developed by Taylor *et al.*[15] The chip device reported here has been designed to isolate axons, but not dendrites or cell bodies from central neurons. The advantage of this chip device over the abovementioned methods is its capability to produce axons in sufficient quantities for conducting systematic analyses, at both the mRNA and protein level. The high productivity of this device occurs because each device contains 872 axon bundles, which are guided to grow into four separate areas that altogether account for 6% of the total chip surface, and from these areas axons are harvested later. This should be compared with the tens of axon bundles guided into two separate chambers when a Campenot type chamber was used[4,35] and the ~200 axon bundles guided into a single chamber using a microfluidic platform as reported by Taylor *et al.*[15] The ease of fabricating the device described here and the simplicity of the procedures used for growing neurons on this device make it feasible to grow neurons on a very large number of chips at the same time. The novel design of straight grooves cut on the backside of glass chips further offers a timesaving and efficient way to harvest the axons from a large number of chips. With all of these technical advantages, the use of the chip device described here will ensure a continuous supply of large quantities of pure axons.

Conclusion

We present here a chip device for producing large quantities of neuronal axons free of neuronal somatodendritic or astroglial contaminations. The RNA and protein components of the isolated axons are then available for detailed biochemical analysis. The use of this chip device should fulfil the demands that are present when conducting proteomic studies of the axon.

The chip device reported here has been designed so that it can be modified easily and used to address different issues in terms of the functional proteome of the axon. For example, the Region 2 area can be coated with molecules implicated in various axonal functions, such as those inducing axons to differentiate into presynaptic terminals and those attracting or repelling growing axons (Supplementary Fig. 2A in the ESI†). Changes in the biochemistry of the axons growing over the Region 2 areas coated with such molecules can then be analyzed in detail using the procedures described herein. Alternatively, neurons can be grown on chips fabricated using the same procedures as described in Fig. 1A except for omitting the step of printing the Region 2 areas, *i.e.*, step 3 in Fig. 1A (Supplementary Fig. 2B in the ESI†). On the surface of such chips, the axons growing along the PLL-coated fine lines will move over grooves 1 and 2 at the backside and stop at ~50 μm beyond the edge of groove 2. Upon cleaving the chips along these grooves, chip fragments respectively containing the growth cone and shaft segments of axons can be obtained. These axonal substructures can thus be harvested separately and also subject to detailed biochemical analyses. Furthermore, when neurons are grown on this device, large numbers of axons are arranged as arrays of parallel bundles that are 850 μm long; these are readily accessible to physically inflicted damage and biochemical treatments from above (Supplementary Fig. 2C in the ESI†). This unique feature will make the device a useful tool when studying the degeneration processes of the axon segments distal and proximal to an injury site and the regeneration process of injured axons. The effects of various extrinsic molecules on the degeneration of the distal axon segments could also be studied using chip devices where the Region 2 surface is coated with such molecules. With these diverse applications, the chip device reported here becomes a powerful platform for studying the molecular mechanisms underlying the various functions of the axon and also for the study of the molecular responses of axons under pathological conditions.

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