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Axon Length Quantification Microfluidic Culture Platform for Growth and Regeneration Study

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Abstract

To fully understand how external biomolecular environment influences axon growth, a method that can easily quantify the extent of axon growth as well as locally control their biomolecular environment is critically needed. Here, we describe a microfluidic culture platform capable of isolating CNS axons from neuronal somata for localized biomolecular manipulation as well as providing linearly guided axon growths for simple and easy quantification of the axon growth length. The axon isolation and guidance capability combined with the multi-compartment configuration make this platform ideal for investigating and screening drugs or other molecular factors that promote axon growth as well as regeneration.

Keywords

Neuron culture microsystem; compartmentalized culture; axon isolation; axon guidance; axon growth quantification; localized biomolecular treatment

1. Introduction

Adult mammalian central nervous system (CNS) cannot be functionally recovered after injury mainly due to the inability of the damaged CNS axons to regenerate. It has been thought that extrinsic environmental factors such as myelin-associated inhibitors are the dominant factor that prevent CNS axons from regenerating^{1, 2}. However, it has been shown that manipulation of axon growth pathways can also promote CNS axon regeneration after injury³. This suggests that not only the environmental factors but also the growth capability of the neurons take important role in axon regeneration.

In order to better understand the growth or regeneration mechanism of axons and to find therapeutic treatments that enhance axon growth or regeneration, methods capable of providing controlled and localized biomolecular microenvironments while allowing easy axon growth quantification are necessary. However, conventional culture plate-based *in vitro* neuron culture methods are limited in providing such features. First, in most conventional

culture methods other than the ‘Campenot chamber’⁴, both neuronal somata and axons are exposed to the same biochemical environment, which hinders investigating localized effects of biomolecular treatments independently. Second, tracking and quantitatively analyzing the extent of axon growth in response to these factors over long culture period at commonly used *in vitro* culture density is challenging. Most of axon growth studies are often performed at a significantly lower cell density (3–20 cells/mm²) and for only a short period of time after cell seeding (< 5 days)^{5–10}. This is largely due to difficulties in tracking and analyzing the length of randomly grown axons.

Several compartmentalized neuron culture microsystems capable of isolating axons from neuronal somata or dendrites have been previously introduced by us and others^{11–13}. However, the growth direction of axons was not controlled and axons formed intensively tangled network upon isolation, making it almost impossible to track or quantitatively analyze individual axons. Therefore, an *in vitro* neuron culture platform that provides axonal isolation with a capability to easily quantify axonal growths could provide important advances in finding biochemical factors or pharmaceuticals that promote the growth capability of CNS axons.

In this chapter, we describe the fabrication and preparation steps for a neuron culture platform where axons are physically guided to grow in straight lines and can be fluidically and physically isolated from neuronal somata and dendrites into six satellite axon compartments for localized biomolecular treatments¹⁴. The neuron culture platform is composed of two poly(dimethylsiloxane) (PDMS) layers and a PDMS ridge structure (Figure 1A). The top PDMS layer is composed of one soma compartment and six axon compartments that are 800 µm apart. The ridge structure that is assembled at the center of the soma compartment reduces the number of required neurons by minimizing the effective cell culture area and increases the axon isolation efficiency. The array of shallow axon-guiding microgrooves (3 × 20 × 10000 µm³) patterned on the bottom PDMS layer has two functions. First, it connects the soma compartment and the axon compartments. The microgrooves confine neuronal somata inside the soma compartment while allowing axons to pass through for isolation inside the six axon compartments (Figure 1B). Second, the microgrooves guide axons to grow in straight lines for easy quantitative axonal length analysis (Figure 1C). Therefore, quantitative analysis of the axon length in response to different localized biomolecular treatments can be performed.

The culture platform introduced in this chapter not only can be used for studying the axonal growth upon localized biomolecular treatments but can also be utilized for studying axonal transection and regeneration, axonal transport under tightly controlled biochemical environments, or studying differential effects of a molecule on somata and axons. Also, the design can be modified to have increased number of axon compartments for achieving higher-throughput.

2. Equipment and Materials

2.1. Equipment

- CNC milling machine, digital balance, laboratory oven, mask aligner, spin-coater, hot plate, oxygen plasma chamber, autoclave, vacuum connected desiccator, humidified 5% CO₂ incubator

2.2. Materials

2.2.1. Culture platform

- 3 inch silicon wafer (SQI Inc.)
- SU-8™ 2002 photoresist (Microchem)
- SU-8™ developer, (propylene glycol methyl ether acetate (PGMEA), Microchem)
- Transparency film mask (CAD/Art service)
- Isopropyl alcohol (EMD Millipore)
- Sulfuric acid (EMD Millipore)
- Hydrogen peroxide (BDH Chemicals)
- (tridecafluoro-1,1,2,2-tetrahydrooctyl)-Trichlorosilane (United Chemical Technologies, Inc.)
- Poly(methyl methacrylate) (PMMA) block (McMaster-Carr)
- Poly(dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning)
- Sharpened stainless steel punch (Technical Innovations, LLC)
- Poly-D-lysine (PDL) (Sigma-Aldrich)
- Calcein-AM (Invitrogen)
- Weighing boat (VWR International)
- Disposable stir rod (e.g. plastic fork)
- Razor blades (VWR International)
- Parafilm (VWR International)
- Disposable petridish (VWR International)

2.2.2. Neuron culture

- Sterilized petridish, forceps, and scissors
- NBB27 with glutamate (DIV 0 – DIV 4): Neurobasal medium (100 ml, Invitrogen), B27 supplement (2 ml, Invitrogen), L-glutamine (2 mM, Sigma-Aldrich), L-glutamic acid (25 µM, Sigma-Aldrich), N-acetyl cysteine (63 ng/ml, Sigma-Aldrich), penicillin/streptomycin (2 ml, Invitrogen)

- NBB27 (from DIV 4): Neurobasal medium (100 ml, Invitrogen), B27 supplement (2 ml, Invitrogen), GluMAX (0.5 mM, Invitrogen), N-acetyl cysteine (63 ng/ml, Sigma-Aldrich), penicillin/streptomycin (2 ml, Invitrogen)

3. Methods

3.1. Culture platform fabrication

3.1.1. Bottom layer fabrication (Figure 2)

1. Clean a silicon wafer with Piranha solution ($\text{H}_2\text{O}_2:\text{H}_2\text{SO}_4 = 1:3$) at 180°C for 20 minutes (*see Note 1*).
2. Thoroughly rinse Piranha cleaned wafer with DI water and dry it with pressurized nitrogen gas.
3. Spin-coat SU-8TM 2002 photoresist on the cleaned silicon wafer at 750 rpm for 30 seconds to obtain a film thickness of 3 μm .
4. Soft-bake the SU-8TM spin-coated wafer at 95°C for 5 minutes on a leveled hot plate.
5. Expose the wafer through a transparency film mask (20,000 dpi) having the microgroove array and align mark patterns with the dosage of 105 mJ/cm^2 .
6. Post-exposure bake the wafer at 95°C for 5 minutes on a leveled hot plate.
7. Develop the wafer with SU-8TM developer (PGMEA) for 45–60 seconds to remove uncross-linked SU-8TM.
8. Rinse the developed wafer with IPA and dry it with pressurized nitrogen gas (*see Note 2*).
9. Vapor-coat SU-8TM patterned silicon wafer with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-trichlorosilane for 20 minutes inside a vacuum chamber, followed by a brief rinsing step with IPA (*see Note 3*).

3.1.2. Top layer fabrication (Figure 3)

3.1.2.1 PMMA master mold fabrication

1. Design one soma compartment and six satellite axon compartments that are 800 μm apart from the soma compartment using any computer-aided design (CAD) program (*see Note 4*). The distances can be adjusted based on the user's need.
2. Pattern the compartment structures on the poly(methyl methacrylate) (PMMA) block using a computer numerically controlled (CNC) milling machine.
3. Rinse the PMMA master mold with IPA 3 times.

3.1.2.2. PDMS master mold fabrication

1. Thoroughly mix PDMS prepolymer with curing agent (10:1 w/w) in a weighing boat using a stir rod (*see Note 5*).

2. Place the PMMA master mold in a disposable container and slowly pour the PDMS mixture until the PDMS reaches 3–5 mm above the PMMA master mold surface.
3. Remove excessive bubbles in the PDMS mixture by placing the PDMS-poured PMMA master mold in a vacuum chamber for 10–15 minutes.
4. Place the PDMS-poured PMMA master mold inside a leveled drying oven and cure at 85°C for at least 3 hours.
5. After PDMS has been fully polymerized, detach the PDMS master mold from the PMMA master mold (*see* Note 6).
6. Vapor-coat the detached PDMS master mold with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-trichlorosilane for 20 minutes inside a vacuum chamber, followed by a brief rinsing step with IPA.

3.1.2.3. Compartment layer fabrication

1. Thoroughly mix PDMS prepolymer with curing agent (10:1 w/w) in a weighing boat using a stir rod.
2. Remove excessive bubbles in the PDMS mixture by placing it in a vacuum chamber for 10–15 minutes (*see* Note 7).
3. Place the PDMS master mold in a disposable container and slowly pour the PDMS mixture until the PDMS reaches 2–3 mm below the top of the PDMS master surface.
4. Remove excessive bubbles in the PDMS mixture by placing the PDMS-poured PDMS master mold in a vacuum chamber for 10–15 minutes.
5. Place the PDMS-poured PDMS master mold inside a leveled drying oven and cure at 85°C for at least 3 hours.
6. Detach the final PDMS top layer from the PDMS master mold and cut out rough boundaries with a razor blade to make the bottom surface perfectly flat.

3.1.3. Ridge structure fabrication

1. Thoroughly mix PDMS prepolymer with curing agent (10:1 w/w) in a weighing boat using a stir rod.
2. In order to obtain a PDMS slab with approximately 2 mm thickness, pour 10 ml of PDMS mixture to a petridish.
3. Remove excessive bubbles in the PDMS mixture by placing the petridish in a vacuum chamber for 10–15 minutes.
4. Place the PDMS-poured petridish inside a leveled drying oven and cure at 85°C for at least 3 hours.
5. Cut out a PDMS ridge structure using a round-hole punch.

3.2. Device assembly and neuron culture preparation steps

1. Place a top layer, a ridge structure, and a bottom layer (surfaces to be bonded facing up) inside the oxygen plasma chamber.
2. Turn on vacuum of the plasma chamber for 2 minutes prior to turning on the plasma and perfuse oxygen gas for 2 minutes upon ignition of the plasma.
3. After the plasma treatment, gently assemble the ridge structure and the top layer onto the bottom layer (*see Note 8*).
4. Immerse the assembled device in a beaker with DI water.
5. In order to remove any air bubbles trapped inside the sealed microgrooves area, degas the device immersed beaker in a vacuum chamber for 5–10 minutes.
6. For sterilization, autoclave the device in water without drying procedure.
7. Take out the device inside a biosafety cabinet and place inside a sterilized petri-dish for easy handling.
8. Remove DI water residue in compartments and apply PDL solution to the soma compartment (800 μ l) and the axon compartments (200 μ l) (*see Note 9*).
9. Coat PDL inside a humidified 37°C incubator overnight.
10. Aspirate PDL solution from all compartments and thoroughly rinse the compartments with sterile DI water 3–5 times with 5–10 minutes interval (*see note 10*).
11. Apply culture medium to the soma compartment (800 μ l) and the axon compartments (200 μ l) and store inside a humidified 37°C incubator overnight before use (*see Note 11*). The device is now ready for cell seeding.

3.3. Microsystem neuron culture

1. Prepare neurons from E16 rat¹⁵.
2. Aspirate culture medium from the soma compartment.
3. Dilute neurons in 200 μ l of NBB27 culture medium with glutamate (cell concentration to match 1000 cells/mm² to the cell culture substrate area) and apply to the soma compartment (*see Note 12*).
4. Incubate the neuron seeded PDMS device inside a humidified 5% CO₂ incubator for 30 minutes to allow neurons to settle down and attach to the bottom surface.
5. Aspirate culture medium from the six axon compartments.
6. Apply additional 600 μ l of culture medium to the soma compartment and add 300 μ l of culture medium to each of the six axon compartment.
7. Replace half of culture medium every 3–4 days (*see Note 13*).
8. NBB27 with glutamate should be changed to NBB27 without glutamate at DIV 4.

3.4. Localized biomolecular treatment

Localized biomolecular treatment either only to the neuronal somata or the isolated axons can be achieved by generating fluidic level difference between the soma compartment and the satellite axon compartments (Figure 4). Minute culture medium flow driven by the hydrostatic pressure from the compartment having higher fluidic level counters diffusion of the treated biomolecules and confines them within the applied compartment.

3.4.1. Localized axon treatment

1. Replace culture medium in the axon compartments with new culture medium (150 μ l) containing biomolecule to be treated (*see* Note 13).
2. Keep culture medium in the soma compartments to be 800 μ l for maintaining fluidic level higher than that of the axon compartments.

3.4.2. Localized soma treatment

1. Replace culture medium in the soma compartment with new culture medium (500 μ l) containing biomolecule to be treated (*see* Note 13).
2. Keep culture medium in the axon compartments to be 400 μ l for maintaining fluidic level higher than that of the soma compartment.

3.5. Axon length analysis

Axons grown inside the culture platform cannot be seen under light microscopes (*i.e.*, bright field, phase contrast) since axons tend to grow along the corners of the axon-guiding microgrooves. Therefore, cells need to be fluorescently labeled using dye such as Calcein-AM.

3.5.1. Axon staining with Calcein-AM

1. Aspirate culture medium from the axon compartment (*see* Note 14).
2. Add 200 μ l of Calcein-AM (1 μ M) diluted in culture medium to axon compartments.
3. Incubate cells inside a humidified 5% CO₂ incubator for 15 minutes.
4. Aspirate the culture medium in both the soma and the axon compartments and rinse 3 times with fresh medium.
5. Add 800 μ l and 300 μ l of medium to the soma compartment and the axon compartment, respectively.

3.5.2. Axon length measurement—When isolated axons are stained with Calcein-AM at DIV 11, linear growth of axons due to the axon-guiding microgrooves can be clearly seen (Figure 5A). Also, it can be seen by the retrograde staining of the dye that most of the neuronal somata having axons inside the axon compartments are in the vicinity of the sealed microgrooves area (Figure 5B). Length of axons can be analyzed by measuring the distance from the sealed microgroove outlets and the end of the axons using any image processing software such as ImageJ (Figure 5C).

4. Notes

1. Piranha cleaning is used to remove organic residues from substrates. Acid piranha is a self-starting reaction which is extremely exothermic. Therefore, the task should always be performed inside a fume hood.
2. When uncross-linked SU-8™ is not properly removed during the development stage, white residue can be observed while rinsing with IPA. In such cases, dry the wafer using pressurized nitrogen gas and further develop in SU-8™ developer (PGMEA).
3. (tridecafluoro-1,1,2,2-tetrahydrooctyl)-trichlorosilane coating allows the replicated PDMS layer to be easily peeled off from the master mold.
4. The side wall that separates the soma compartment from the axon compartments should be designed to have thicker profile on top (Figure 1B-inset) to prevent the PDMS wall from being torn during the final top layer replication process (Figure 3). Alignment marks should be added to the design as appropriate.
5. Improper mixing of polymer base and curing agent can cause unevenly cured PDMS and can contaminate the master mold.
6. Polymerized PDMS surfaces can be easily contaminated by dusts or particles. Wrapping the PDMS devices with parafilm after it has been sufficiently cooled down can prevent contaminations.
7. Intense bubble generation can occur when using a PDMS structure as the replication master mold. Therefore, additional degassing of PDMS mixture prior to pouring it to the PDMS master mold is recommended.
8. Gently assemble the top layer to the bottom layer since sealed microgrooves area can easily be collapsed.
9. In order to properly coat the sealed microgrooves with PDL, generate fluidic level difference between the soma compartment and the axon compartments.
10. PDL inside the sealed microgrooves can be thoroughly rinsed by generating fluidic level difference between the soma compartment and the axon compartments during the rinse process.
11. Incubation of the device allows any potential debris or toxic residue to be removed.
12. Cell numbers can be adjusted according to the experimental design (500–2000 cells/mm² is recommended).
13. Be careful not to damage the cells.
14. Aspirate the culture medium from the outer end of the axon compartments to prevent axons from being damaged.

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Figure 1.

(A) PDMS components of the developed quantitative axon growth analysis neuron culture platform. (B-C) Illustration showing axon isolation as well as axon guidance via the array of shallow microgrooves patterned on the bottom substrate. (B-Inset) Cross-sectional view showing 20° inclined PDMS wall separating the soma and the axon compartment (see Note 4).

Figure 2. Illustrations showing the fabrication process of the bottom PDMS layer by conventional photolithography and PDMS soft-lithography processes.

Figure 3. Illustrations showing the fabrication process of the top PDMS layer by double PDMS soft-lithography process using CNC milling machine engraved PMMA as a master mold.

Figure 4.

Illustrations showing fluidic level difference in the case of localized axon treatment (left) and localized soma treatment (right).

Figure 5.

(A) Linear growth of the isolated/guided axons inside the axon compartment at DIV 11. (B) Close-up view of the retrograde stained neuronal somata inside the soma compartment having axons isolated inside the axon compartment. (C) Measurement scheme of the isolated/guided axon. White dotted lines indicate compartment boundaries.