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Application of Soft Lithography and Micro-Fabrication on Neurobiology

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1. Introduction

Soft lithography should be regarded as a complement to common lithography, providing a low-expertise route toward micro/nanofabrication and playing an important role in microfluidics (YN Xia & Whitesides, 1998). The resolution ranges from 5 to 100 nanometer (Pilnam Kim, et al. 2008). Patterns generated by the soft lithography are transferred repeatedly to the soft flexible materials, and then are printed on the medium substrates. In this field, micro contact printing (μ CP) is the most widely used technique, especially in bioscience research. Combined with microfluidic patterns technology, several kinds of the extracellular matrix proteins like polymers can be printed to make cells grow according to the designed patterns (Tai Hyun Park, et al. 2003). The cell growth, differentiation in vitro can be regulated in the respect of spatial structure of extracellular matrix (Y. Nam, et al. 2004). So the morphology of neural cells and the influence of spatial structure can be investigated on the micron or even nano-scale level substrates. The closed loop of neural cells can be constructed in order to simulate the complex neural network in vivo. Finally, communication with the specific environment in vitro will be achieved by multi-electrode arrays (MEA). Our previous work used μ CP technique can build more solid patterns. By comparing three different extracellular matrixes, PEI can obtain much better results, which adhering more neural cells to form reliable design. However, it is not perfect for the specific neural network construction and the patterned neural cell culture on MEA. In subsequent research, we improved the parameters of the template and achieved a big progress on microfluidic patterning technique to microfabricate patterns. Patterns of biomaterials were constructed with the help of the advanced soft lithography to do the primary cell culture, such as dopaminergic neurons in the substantia nigra and GABAergic neurons in the striatum. Finally, the biocompatibility of MEA was validated initially by primary striatal neuronal culture. Meanwhile, new strategy of structural microfabrication on MEA surface was further explored.

2. Soft lithography materials

Why soft lithography is called "soft"? One of the reasons is the use of soft organic materials such as polydimethyl siloxane (PDMS), polymethyl methacrylate (PMMA), and polycarbonate (PC). PDMS is most widely used in bioscience research, because it has good

biocompatibility, chemical stability, optical transparency, air permeability, elasticity. Moreover, the polymer precursors can be aggregated into a mold by UV radiation. PDMS polymerization is shown in Figure 1.

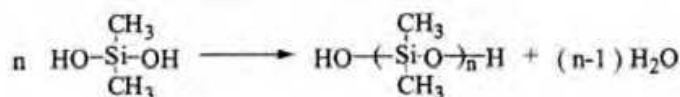


Fig. 1. PDMS polymerization

To cure the PDMS prepolymer in general, a mixture of silicon elastomer and a curing agent (10:1, Sylgard 184 silicone elastomer kit, Dow Corning Corp.) is poured onto the master and placed at 70-80°C for 1 h. The character of the PDMS is closely related to the mixture ratio, curing temperature, and vacuum. Silicon, quartz or glass, and some photoresist are the most common materials to fabricate the masters by standard lithography, transferring the patterns to the PDMS stamp.

3. Soft lithography fabrication methods and it's application in patterning

Various soft lithographic technologies have been applied to fabricate high-quality microstructures and nanostructures including micro contact printing (μCP), replica molding (REM), microtransfer molding (μTM), micromolding in capillaries (MIMIC), and solvent-assisted micromolding (SAMIM). Here, three soft lithographic methods are introduced to fabricate micropatterns onto a surface or MEA: μCP , microfluidic patterning technique and microstencil. The former two can be achieved using the same PDMS stamps and molds. A novel technology was applied to get the high depth-to-width ratio silicon-based mold to fabricate the topographic PDMS microstencil with microfluidic channel (Y.Nam, et al, 2006). Finally, microchannels on MEA with polyimide (PI) guiding the cell growing were also introduced.

3.1 Microcontact printing (μCP)

μCP is a direct method for pattern transfer, generating a non-structured, chemically modified surface. The process of μCP is shown in figure 2. Photolithography was used for the fabrication of silicon-based masters in preparing PDMS stamps. Multi-layer molds were made of thick photoresist like SU-8 on silicon or glass wafers by standard lithography techniques. It was subsequently placed at least 30min in an oven at 160°C to make the photoresist adhere to the substrate closely. Release agent DC20 or OTS were always spin-coated and drying on the master before pouring PDMS. Liquid PDMS (Sylgard-184 from Dow Corning) was poured onto the mold and clamped by the foil, so that the shape of the mold microstructure was transferred to PDMS membrane. It was subsequently placed at least 2 h in an oven at 80°C. The molded PDMS slab was then peeled off and placed onto a glass slide for handling. After curing, PDMS stamps are soaked in a protein "ink", such as PEI, PLL, or LN. 20 minutes later, the "ink" was blew off by using nitrogen gas. Then the raised regions were brought into conformal contact with a substrate in order to print the ink onto the substrate surface. The material of interest was transferred from the PDMS stamp onto the substrate surface. The microscopy of the PDMS stamps were shown in figure 3.

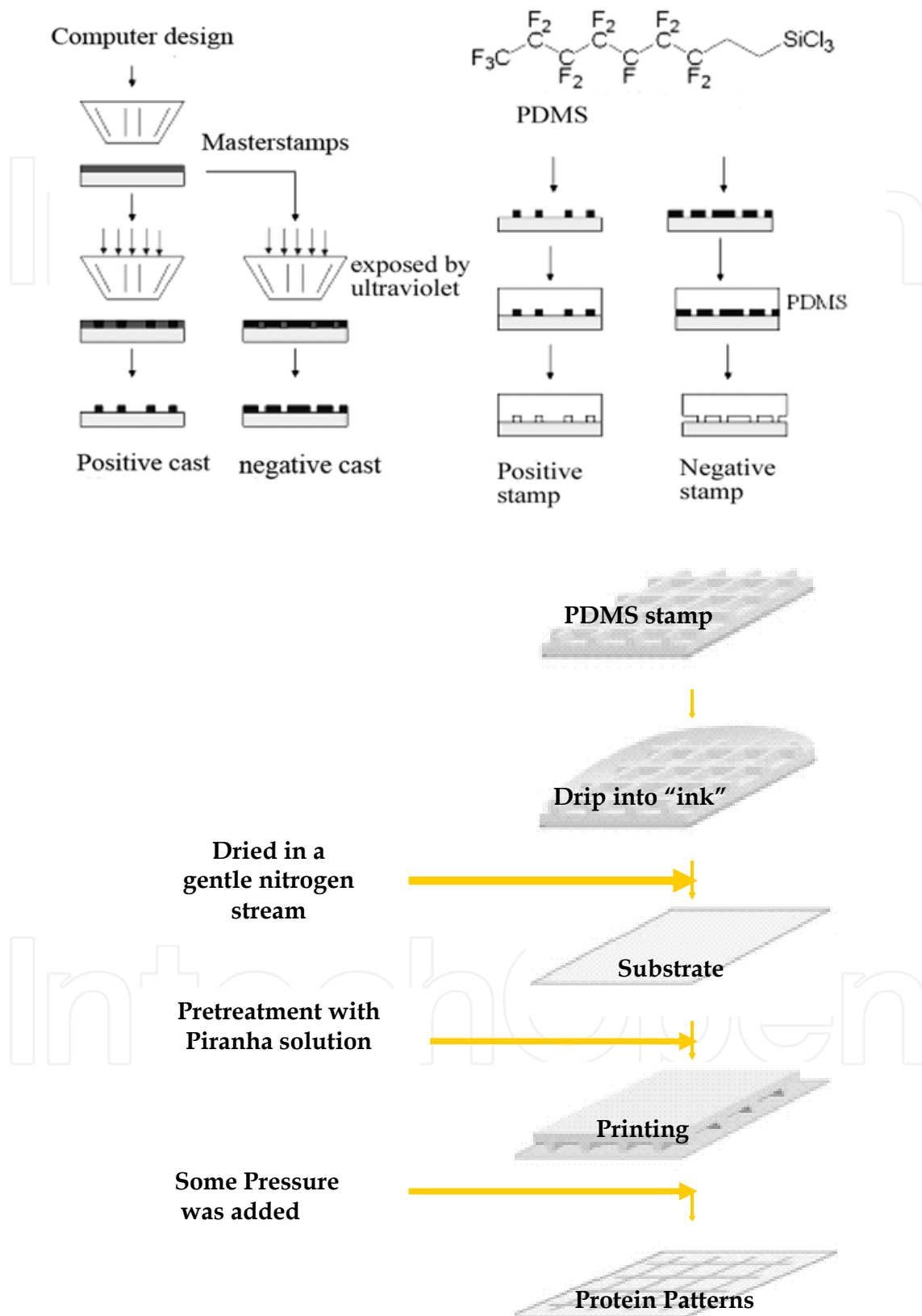


Fig. 2. Schematics of the processes of μCP

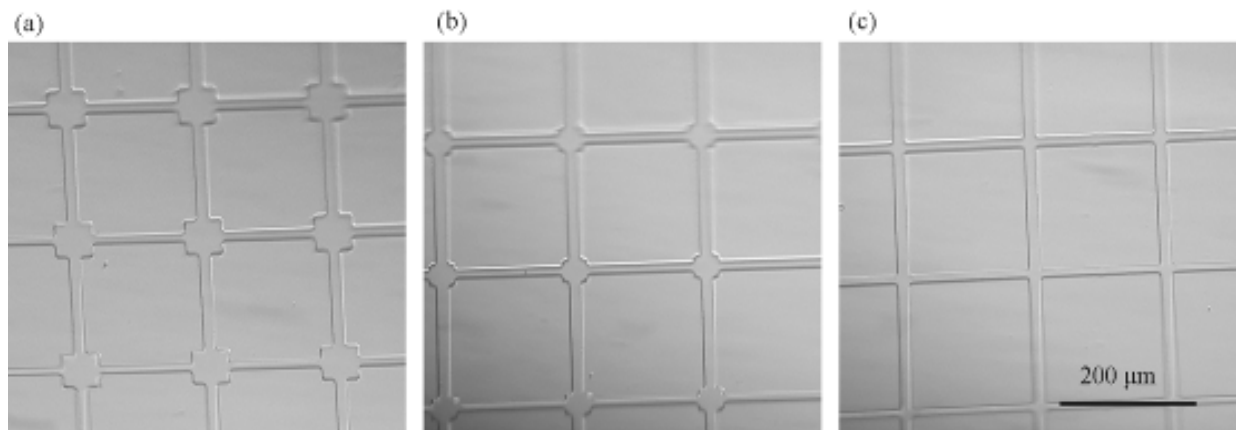


Fig. 3. Microscopy of the PDMS stamps

The bare areas of substrate surface that the PDMS stamp has not touched can be exposed to another coating material. μ CP provides the patterning of self-assembled monolayers (SAMs) of alkanethiols on gold, and the resulting control over the adsorption of adhesive proteins facilitates the patterning of cells on substrates.

μ CP enables easy stamp replication, fast printing using parallelization, and low-cost batch production. A conformal contact between the stamp and the surface of the substrate is the key to its success. The polymer stamps also minimize the problems of sample carry-over and cross contamination. Printing has the advantage of simplicity and convenience: Once the stamp is available, multiple copies of the pattern can be produced using straightforward experimental techniques. Printing is an additive process; the waste of material is minimized. Printing also has the potential to be used for patterning large areas.

However, μ CP has some limitations that are mainly caused by the use of a soft polymer stamp. The swelling of a stamp during inking often results in an increase in the pattern size by diffusion of the excessive printed molecules on the substrate.

3.2 Microfluidic patterning using microchannels

The difference between μ CP and microfluidic patterning is that PDMS stamps are soaked in the “ink” in the former usage, but the stamps contact the substrate forming microchannels delivering the materials for cell adhesion or cell suspension to the desired area in the latter usage because of the elastic nature and hydrophobicity of PDMS. The substrate tilted 45 degree, drop of liquids were injected to the PDMS microchannels by the pipette. Then the substrate was put on the test tube rack vertically for 25 to 30 min. Patterns were formed of after the liquid dried.

While this method has been used primarily for surface attachment of cells, it may be possible to adapt this method to three-dimensional tissue constructs. In many cases three dimensional tissue constructs promote cellular differentiation and more authentic cellular morphology and metabolism.

3.3 Microstencil on MEA

The former two methods enable patterning adhesion molecules and guiding cultured cells grow physically. But the cells' communication and interactions in co-cultures are difficult to be detected, which is important to research the function of the cells network.

MEA was a cell-based biosensor for extracellular electrophysiological investigations of neuronal networks. PDMS microstencil was designed to pattern adhesion molecules at the surface of MEA guiding cultured cells grow along the patterns. PDMS microstencil mold was fabricated by a complex photomask aligning method, shown in figure 4.

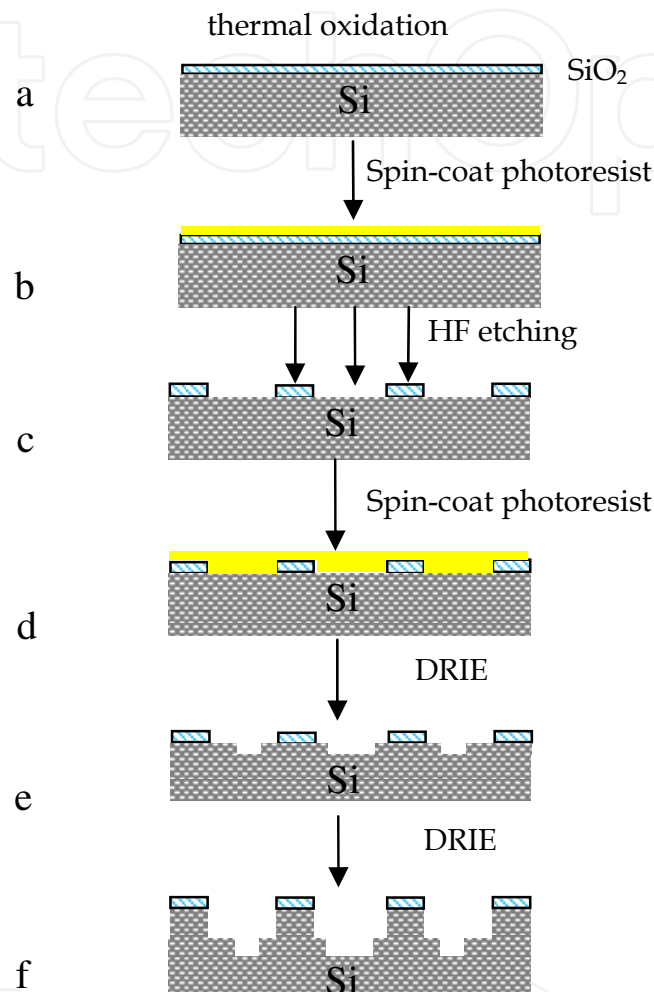


Fig. 4. Schematics of the fabrication of microstencil mold

525 μm silicon was thermal oxidated with 4000 Å SiO₂ (see Fig. 4a). The substrate was first coated with a thin photoresist (AZ AZ9912) for 30 s at 3000 rpm (see Fig. 4b). 8 × 8 SiO₂ arrays were fabricated by wet etching as the RIE mask which was the same as the MEA structure (see Fig. 4c). Photoresist was spin-coated to the silicon with SiO₂ mask again (see Fig. 4d) and selectively exposed to UV under a chromium photomask. The silicon was etched 30 μm by deep reactive ion etching (DRIE) with photoresist in order to construct the microchannel, (see Fig. 5a). Then photoresist was removed by ultrasonication in acetone. The silicon was selectively etched 70 μm by DRIE with SiO₂ resist, forming the topographic PDMS microstencil mold (see Fig. 5b). The high depth-to-width ratio silicon-based mold was designed to penetrate through the PDMS membrane on the MEA to expose the electrodes and form the microchannel between the electrodes so that the MEA could also work.

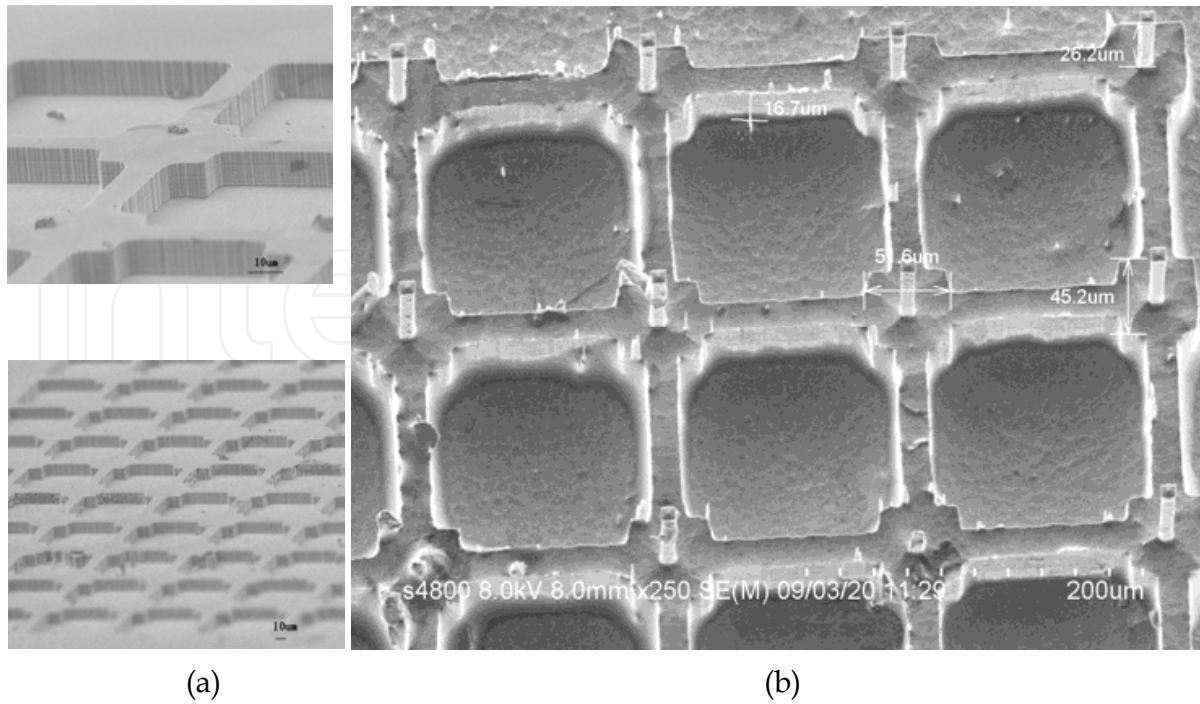


Fig. 5. SEM images of PDMS microstencil mold

The mixture of PDMS prepolymer and curing agent was spin-coated on the mold for 40 s at 4000 rpm. The coated mold was cured for 2 hours at 110°C in a convection oven. The fully-cured PDMS-coated mold was soaked in an acetone ultra-sonication bath until the PDMS layer released from the mold. The detached microstencil was rinsed with IPA and DI water. The upside and downside of PDMS microstencil with microholes and microchannels SEM images was shown in figure 6.

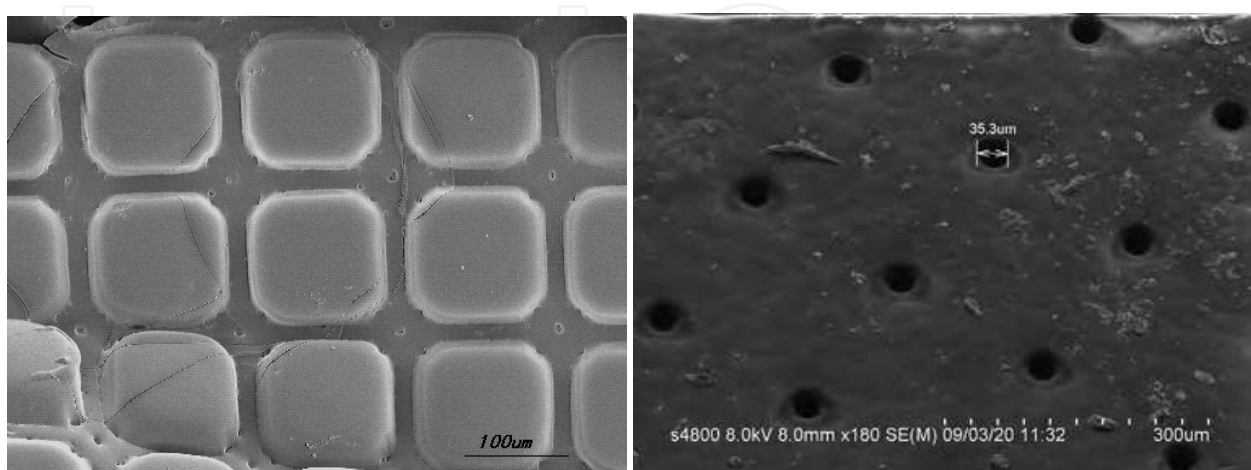


Fig. 6. SEM images of PDMS microstencil

However, PDMS microstencil is difficult to practise because it is hard to align with MEA and the silicon mold is easy to fracture when lifting off.

3.4 MEA with microchannels for patterning

Planar MEA are developed to study electrogenic tissues such as dissociated neuronal cultures (Hiroaki Oka, et al. 1999). They have been widely used with dissociated cultures for a variety of neuroscience investigation including learning and memory and cell-based biosensors for the detection of neurotoxins (Conrad D. James, et al. 2004). But the neurons grow disorderly and cannot form a network so that the function is not the same as the cells in vitro. Combining the patterning technology and MEA forming neuronal networks is the efficient method to research the neurobiology.

μ CP, microfluidic patterning technique and microstencil are difficult to operate because the space is too small and the PDMS stamp or stencil can hardly align with MEA. The best and easiest way to forming neuronal networks is to fabricate the microchannels on MEA with polyimide (PI) guiding the cell growing.

MEA were fabricated using a conventional semiconductor process (Guangxin Xiang, et al. 2007). After cleaning the polished quartz glass wafer, the conductive layer of Au/Ti film (Au 3000 Å and Ti 700 Å) was sputtered. 8×8 electrode arrays were left with the photomask protection by standard photolithography. Then, a combination of SiO₂/Si₃N₄/SiO₂ (3000 Å /4000 Å /3000 Å) passivation layers was deposited onto the substrate using plasma enhanced chemical vapor deposition (PECVD), and the insulating layers on the electrodes and the bonding-pads were removed by inductively coupled plasma (ICP) (see Fig. 7a). Finally, Negative photosensitive polyimide (AP2210B, Fujifilm Electronic Materials Inc) was spin-coated to form microchannels having a thickness of 3~4 μ m and photo-etched by the standard procedure to expose the microelectrodes and the terminals (see Fig. 7b).

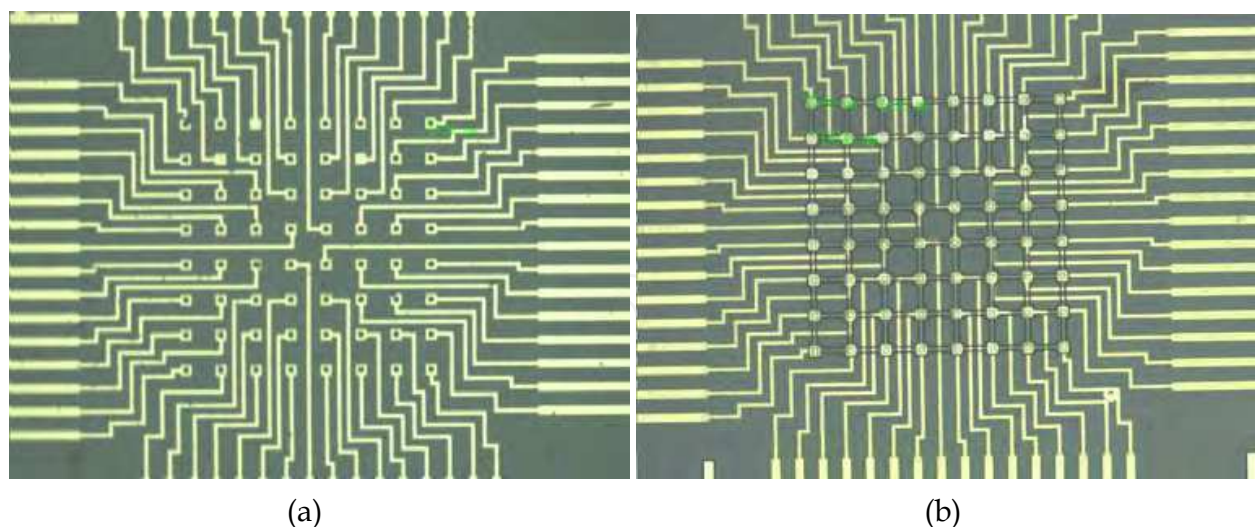


Fig. 7. Microscopy of the MEA with PI microchannels

GABAergic neurons in the striatum and PC12 cells were cultured on MEA with PI microchannels which were coated with poly-L-lysine (PLL) to promote cell adhesion, (see Fig. 8a, 8b). PI microchannels could be seen between the electrodes and the neural cell can grow along the microchannels. However the nerve cell synapse could not formed along the microchannels. Because the depth of microchannels could not match the neurons and the

PLL could not be guarantee to coat the microchannels effectively after days. There is still a lot of work to do to construct the neuronal networks on MEA to study the cells function as in vitro.

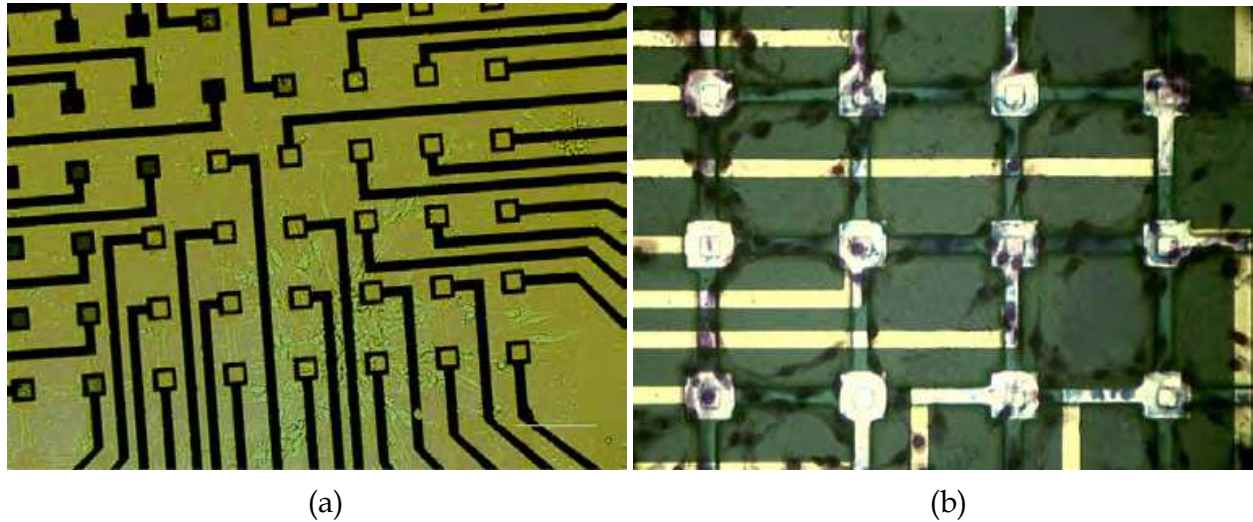


Fig. 8. Microscopy of the cells cultured on MEA with PI microchannels (a) GABAergic neurons in the striatum cultured at 3 days (b) PC 12 cells cultured at 6 days

4. Construction of neural network by applying soft lithography

A man-made neural network on electrode can be applied to do basic research of neuroscience, be a kind of biosensor for drug discovery [9] and even be implanted into brain to establish artificial connections that could form the basis of a neural prosthesis[10]. These fields have caused much attention in the world [11]. In the past, there are three strategies to realize a simplified neural network in vitro, such as mechanical fixation-applying spatial restrictions[12,13], physical modification of surface roughness and surface topography[14,15], chemical polymer microfabrication on surface – using soft lithography. Soft lithography is created by Whitesides in 1993[16]. It is used to create chemical structures on surfaces, including μ CP, μ FN and other downstream techniques. These microfabrication techniques that control both the size and shape of the cell anchored to a particular surface are extremely useful for understanding the influence of the cell-material interface on the behavior of cells [17, 18]. The adhesion and survival of neural cells should be considered firstly for the patterned neural cell culture in vitro. So the selection of appropriate cell-attracting substances is an important step for pattern design in micro-contact printing. Ideal substances encourage good cell-substrate interactions, constantly stimulate the cells by substrate-bound chemical, biological, electrical or mechanical signals [19, 20] and even regulate neuritis growth on designed patterns. The most commonly used coating reagents to promote cell adhesion are extracellular matrix (ECM) proteins like laminin (LN), positively charged polymers such as poly-l-lysine (PLL) and synthetic amide-linkage-free compounds such as polyethylenimine (PEI). Therefore, the characteristics of three different substrates, PEI, PLL, LN were compared by the primary neuron culture in our previous work. The PEI characterized with strong positive surface charges was validated to fabricate more continuous and integrated micro-contact printing neural patterns under serum-free culture conditions than PLL, LN[21].

For the functional neural network construction on MEA, an inevitable question that should be addressed finally is how to realize accurate opposite between neurons and the electrode under neurons. We assume firstly microfluidic technique may have more advantages than μ CP. In subsequent research, we achieved satisfied patterns by microfluidic technique for further research with the help of the progress on parameters of template. Specific neural network were constructed by applying advanced soft lithography above to do the primary cell culture, such as dopaminergic neurons in the substantial nigra and GABAergic neurons in the striatum. The conditions of neuronal adhesion on different patterns (grids and lines) were also observed using several techniques, including atomic force microscopy, immunohistochemistry, transmission electron microscope and scanning electron microscope.

4.1 Neural network with rat fetal hippocampal cells by μ CP patterns

In previous study, we examined the ability of another positively charged polymer, polyethyleneimine (PEI), to promote neuronal adhesion, growth and the formation of a functional neuronal network in vitro. PEI, PLL and LN were used to produce grid-shape patterns on glass coverslips by μ CP. Post-mitotic neurons from the rat fetal hippocampus were cultured on the different polymers and the viability and morphology of these neurons under serum-free culture conditions were observed

4.1.1 Cells adhesion

The number of cells that adhere to the different substrates after 24 h in culture is shown in Fig. 9. The adhesive effects were evaluated by calculating the ratio of cell numbers that adhere to the grid-like patterns divided by the total area of printed polymer. We found that the positively charged polymers (PLL and PEI) had a significantly higher level of cell attachment than LN ($p < 0.05$)[13].

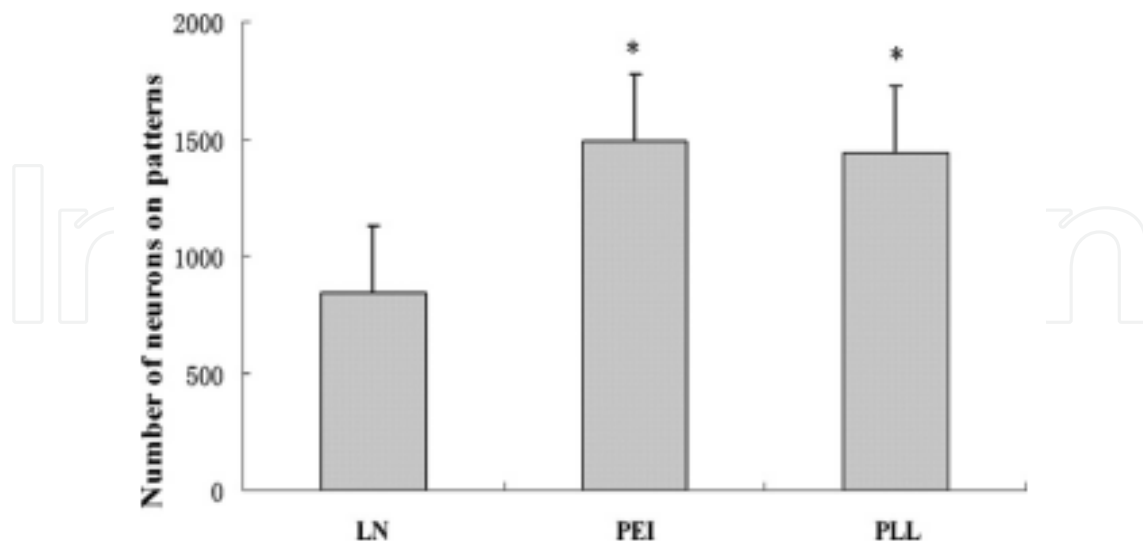


Fig. 9. The adhesive effects were test by analysis of the number of neurons on the area (mm^2) of LN, PEI and PLL grid patterns after 24 h in culture. The asterisks indicated neurons on PEI and PLL patterns had significantly higher lever than on LN patterns, $n = 12$, $p < 0.05$.

4.1.2 Cells viability

In Fig.10, We show that neurons cultured on the PEI- and PLL-coated surfaces adhered to and extended neurites along the grid-shape patterns, whereas neurons cultured on the LN-coated coverslips clustered into clumps of cells. In addition, we found that the neurons on the PEI and PLL-coated grids survived for more than 2 weeks in serum-free conditions, whereas most neurons cultured on the LN-coated grids died after 1 week[13].

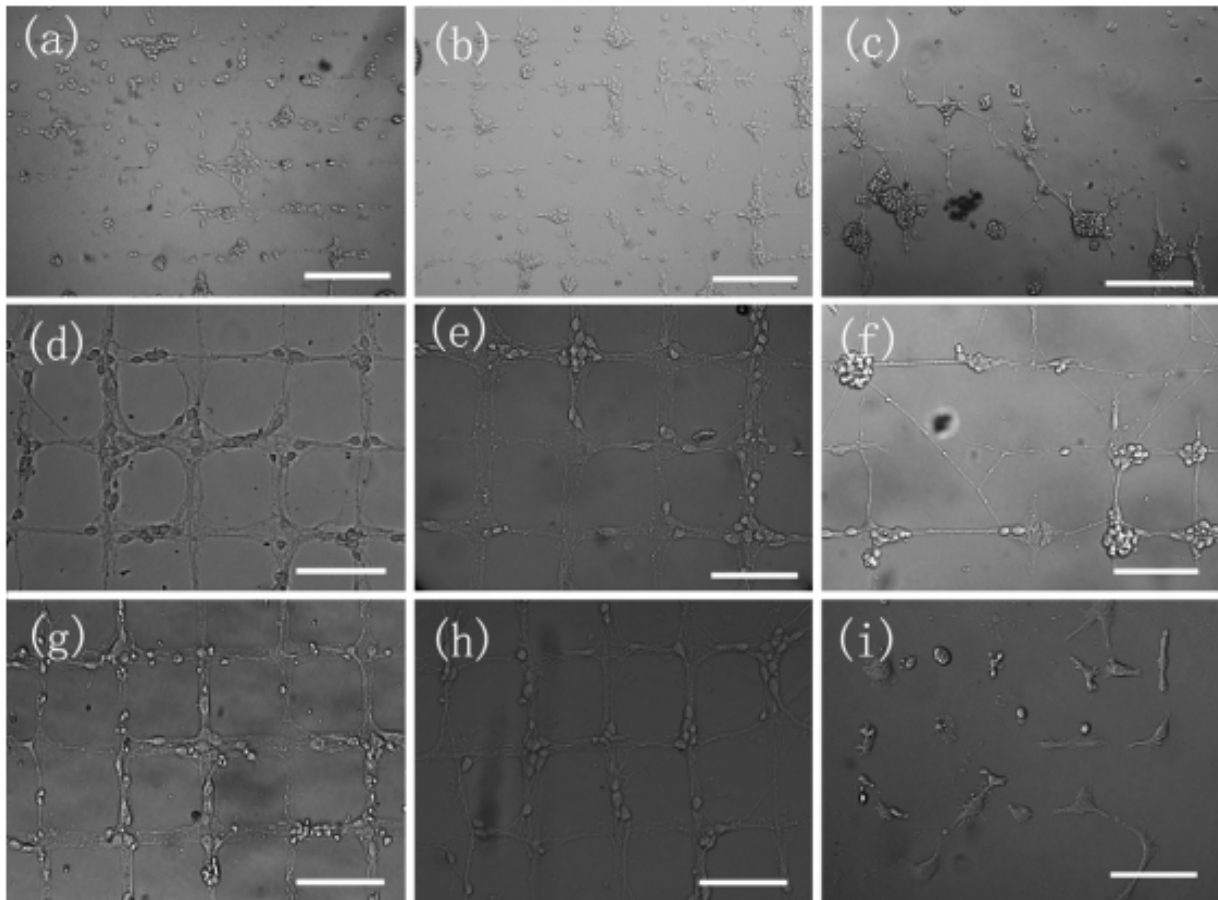


Fig. 10. Images obtained using the phase contrast microscope, showing cells cultured on PEI, PLL and LN polymeric films at different time points. (a, d, and g) Representative images of neurons cultured on PEI grid patterns at 24 h, 7 days, and 14 days, respectively, show that neurons adhere and grow accurately along the PEI grids at differently time points. (b, e, and h) Representative images of neurons cultured on PLL grid patterns at 24 h, 7 days, and 14 days, respectively. (c, f, and i) Representative images of neurons cultured on LN grid patterns at 24 h, 7 days, and 14 days, respectively. The images (c and f) show that the neurons on the LNgrid patterns often accumulate at the cross points of the grids. The image (i) shows that most neurons disappear after 14 days in culture and only small areas of the neural cells exist. (a-c) Bar = 100 μm ; (d-i) bar = 50 μm .

4.2 Specific neural network with two relative neurons, such as dopaminergic neurons and GABAergic neurons

There is a closely relationship in the respect of function and structure between dopaminergic neurons in the substantial nigra and GABAergic neurons in the striatum. As well-known,

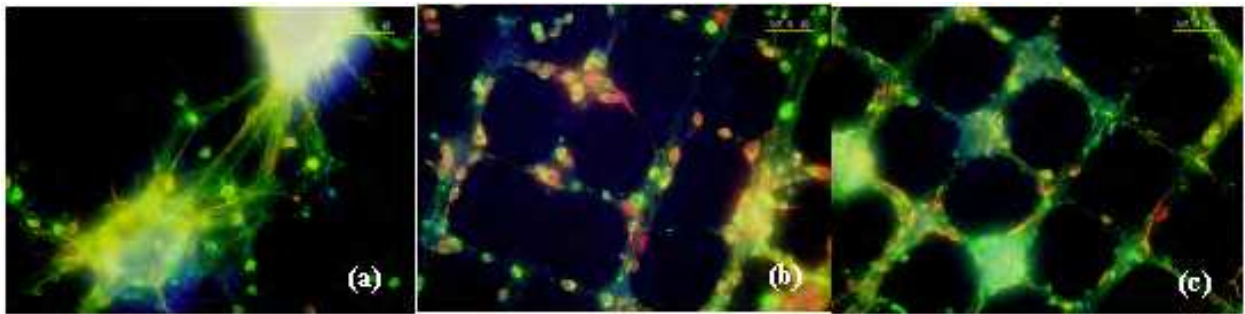
Parkinson's disease is due to the loss and injuries of dopaminergic neuron in substantia nigra which cause a decrease in nerve fibers projected to the new striatum. Finally, reasonable synaptic connections and neural network can not be established. Therefore, it is expected to establish cell models to investigate the relationship between these two kinds of coherent neurons and construct an artificial neural network in vitro by the application of soft lithography. In present work, specific neural network with dopaminergic neurons and GABAergic neurons co-culture was established by μ CP PEI grid patterns. Meanwhile, PEI was validated again to fabricate more continuous and integrated neural patterns by using μ CP and microfluidic technique both.

4.2.1 Neural network by μ CP

Different kinds of neural network by μ CP were established with neuron from the striatum, dopaminergic neurons from the substantia nigra and both of them co-culture. The conditions of neuronal adhesion on different pattern figures were observed using several techniques, including immunocytochemical staining, transmission electron microscope and scanning electron microscope. Using immunocytochemical staining, transmission electron microscope, we identified the types of neural cells and observed some neurosynapse-like structures near the neuronal soma on PEI-coated coverslips. These findings indicate that PEI is a suitable surface for establishing a functional neuronal network in vitro.

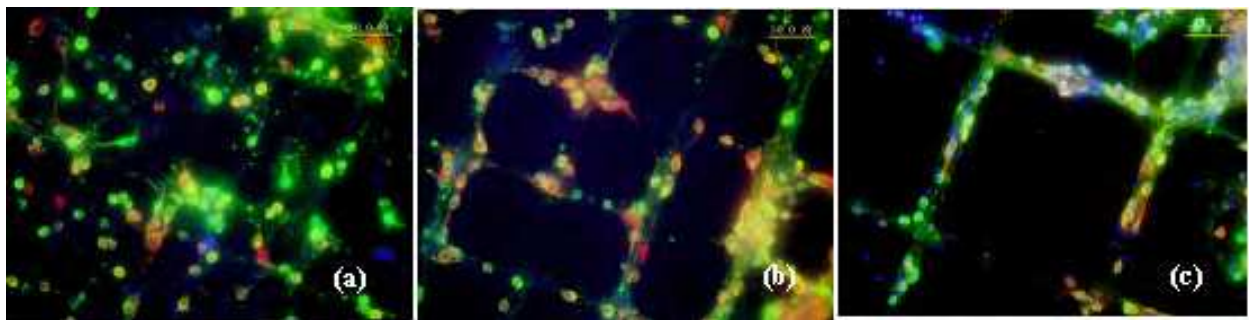
4.2.1.1 Investigation of neural cell types and neurite elongation along the grid-like patterns by immunocytochemical staining and SEM

PEI, PLL and LN were used to produce grid-shape patterns on glass coverslips by micro-contact printing. GABAergic neurons and medium spiny neuron from the rat striatum, dopaminergic neurons from the rat substantia nigra and both of them co-culture were researched separately on the different polymers coated surface. The viability and morphology of these neurons under serum-free culture conditions were observed using fluorescent microscopy in Fig. 11, Fig. 13, Fig. 14. After 7 days in culture, we found that the neural cell bodies on the PEI patterns were located mostly at the cross-points of the grid, whereas neurites extended along the line of the grid-like patterns. More continuous and integrated neural network was achieved finally. On the PLL-coated coverslips, the neural patterns appeared to be integrated. But several cells clustered at the cross-points of the grid disappeared gradually after the media was replaced. In contrast, cells cultured on the LN-coated grids were generally clustered into clumps and cannot form satisfied patterns. Different sizes of PEI pattern were produced by microcontact printing. In Fig. 12, compared with $50\ \mu\text{m}$, $100\ \mu\text{m}$, $200\ \mu\text{m}$ pattern sizes, we found few difference early. After 7 days or 14 days culture, most neural cells on $200\ \mu\text{m}$ size grew well and were seldom found to overlap each other, unlike those on $50\ \mu\text{m}$ size clustered into clumps at the cross-points of grid and disappeared gradually. Identified with immunocytochemical staining, we found that neural cells from the rat substantia nigra were TH positive, synaptic vesicle protein were synaptophysin positive in Fig. 13 and cells from the rat striatum were GABA positive or DARPP-32 positive in Fig. 11, Fig. 12, Fig. 14. SEM show neurons outgrowth on PEI-coated patterns and validate the findings by immunocytochemical staining in Fig. 15, Fig. 16.



(a) on LN grid patterns (b) on PEI 100 μ m grid patterns (c) on PLL 100 μ m grid patterns bar=50 μ m

Fig. 11. Immunofluorescent image of anti-GABA (green fluorescence) +anti-MAP2 (red fluorescence) labelled striatal neurons cultured for 7 days on different substrates, the nuclei of neurons were stained with Hoechst x400



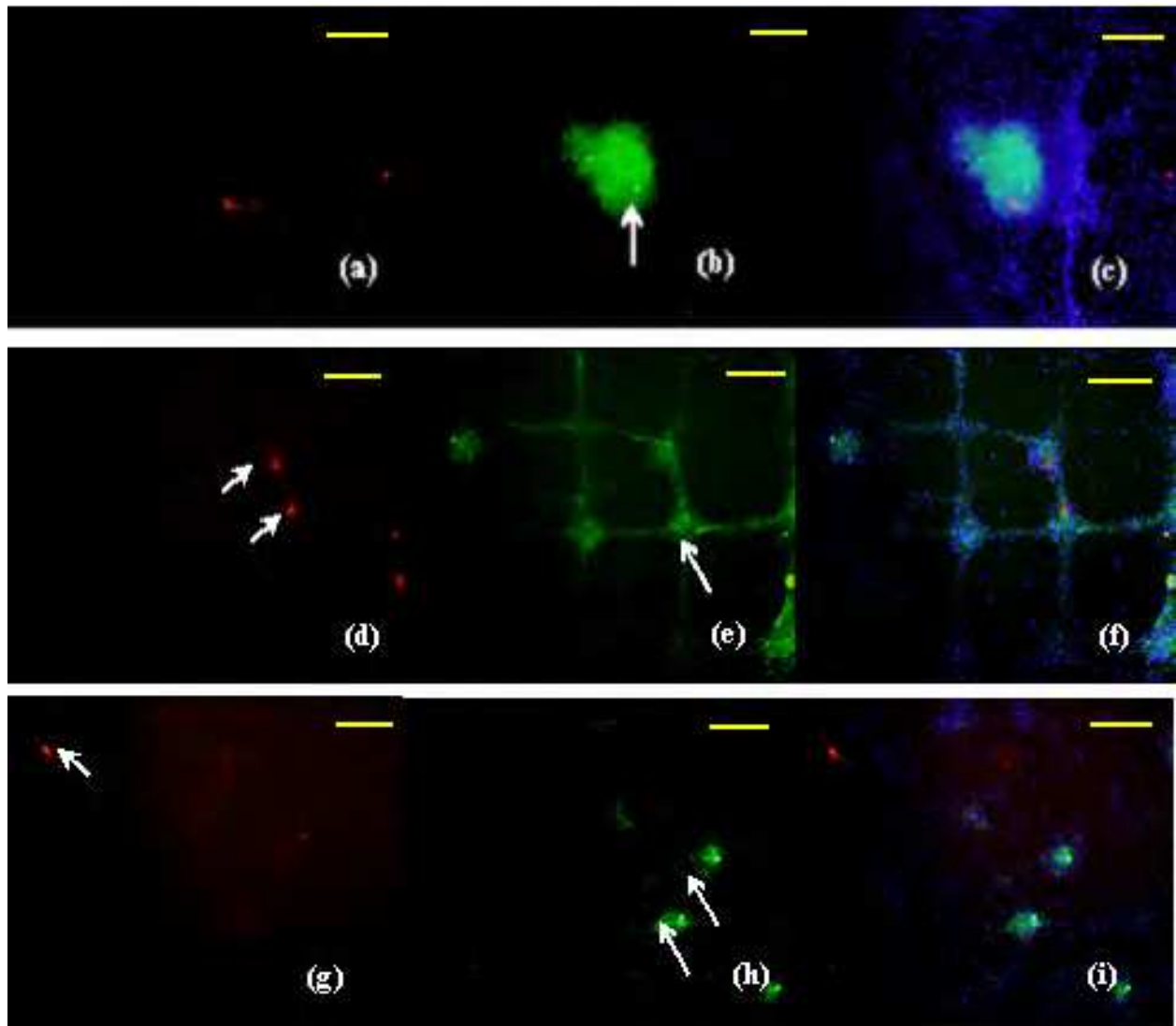
(a) on 50 μ m patterns (b) on 100 μ m patterns (c) on 200 μ m patterns bar=50 μ m

Fig. 12. Immunofluorescent image of anti-GABA (green fluorescence) +anti-MAP2 (red fluorescence) labeled striatal neurons cultured for 7 days on different sizes of PEI patterns, the nuclei of neurons were stained with Hoechst x400



(a) on LN grid patterns (b) on PEI 100 μ m grid patterns (c) on LN+PEI bar=20 μ m

Fig. 13. Immunofluorescent image of anti-TH (red fluorescence) labeled dopaminergic neurons from the substantia nigra and anti-synaptophysin (green fluorescence) labeled synaptic vesicle protein cultured for 7 days on different substrates. x200



(a-c) co-culture neurons growing on LN grid patterns. (a) Immunostaining with anti-TH (red fluorescence) labelled dopaminergic neurons. (b) Immunostaining with anti-DARPP-32 (green fluorescence) labeled medium spiny neuron from the striatum (arrow). (c) Merged image of (a) and (b), showing TH positive neurons on the LN-coated patterns actually adhere to the cluster formed by medium spiny neuron. (d-f) co-culture neurons growing on PEI grid patterns. (d) Immunostaining with anti-TH (red fluorescence) labelled dopaminergic neurons (arrows). (e) Immunostaining with anti-DARPP-32 (green fluorescence) labeled medium spiny neuron from the striatum (arrow). (f) Merged image of (d) and (e) showing that DARPP32 positive neurons cultured on the PEI-coated patterns form a continuous and integrated neural network, and two TH positive neurons adhere to the cross-points of grid. (g-i) co-culture neurons growing on PLL grid patterns. (g) Immunostaining with anti-TH (red fluorescence) labeled dopaminergic neurons (arrow). (h) Immunostaining with anti-DARPP-32 (green fluorescence) labeled medium spiny neuron from the striatum (arrows). (i) Merged image of (g) and (h) showing only two DARPP32 positive neurons adhere to the cross-points of grid. TH positive neurons were not restricted by the grid pattern. bar = 100 μ m

Fig. 14. Immunofluorescent image of anti-TH labelled dopaminergic neurons from the substantia nigra and anti-DARPP32 labeled medium spiny neuron from the striatum co-culture for 7 days on different substrates, the nuclei of neurons were stained with Hoechst x200

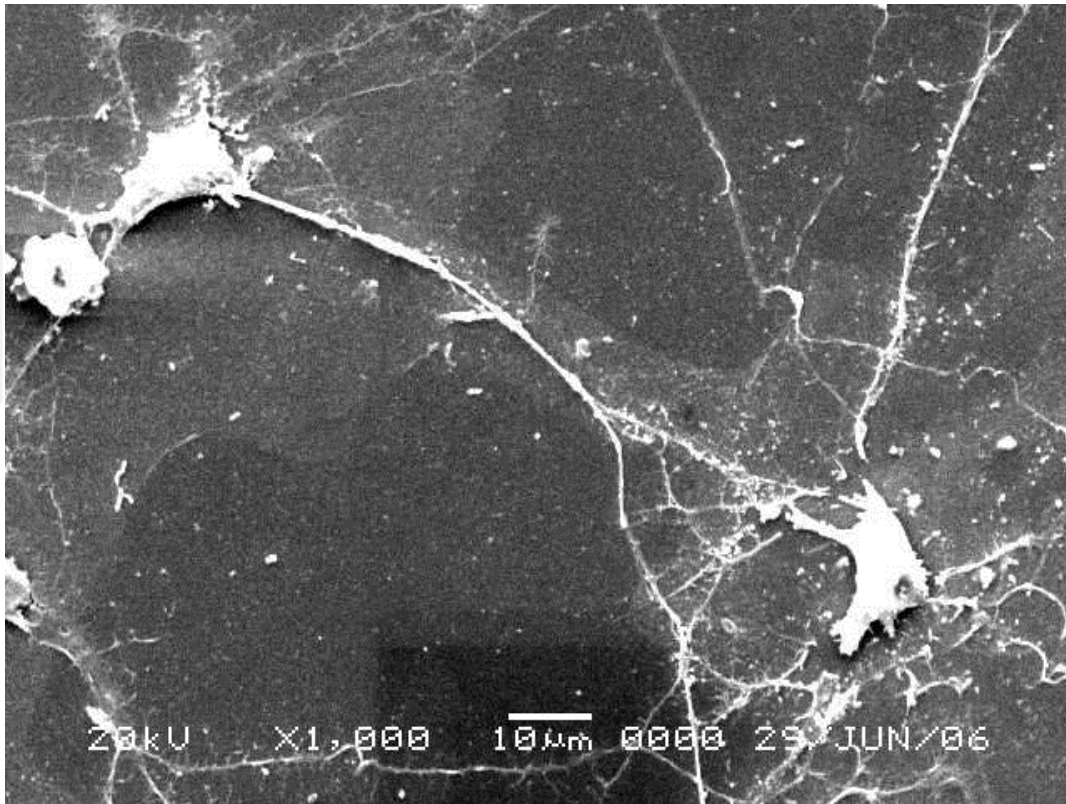


Fig. 15. SEM show neurons outgrowth on PEI-coated patterns. Somata of neurons located on the cross point and neurites extend along the lines

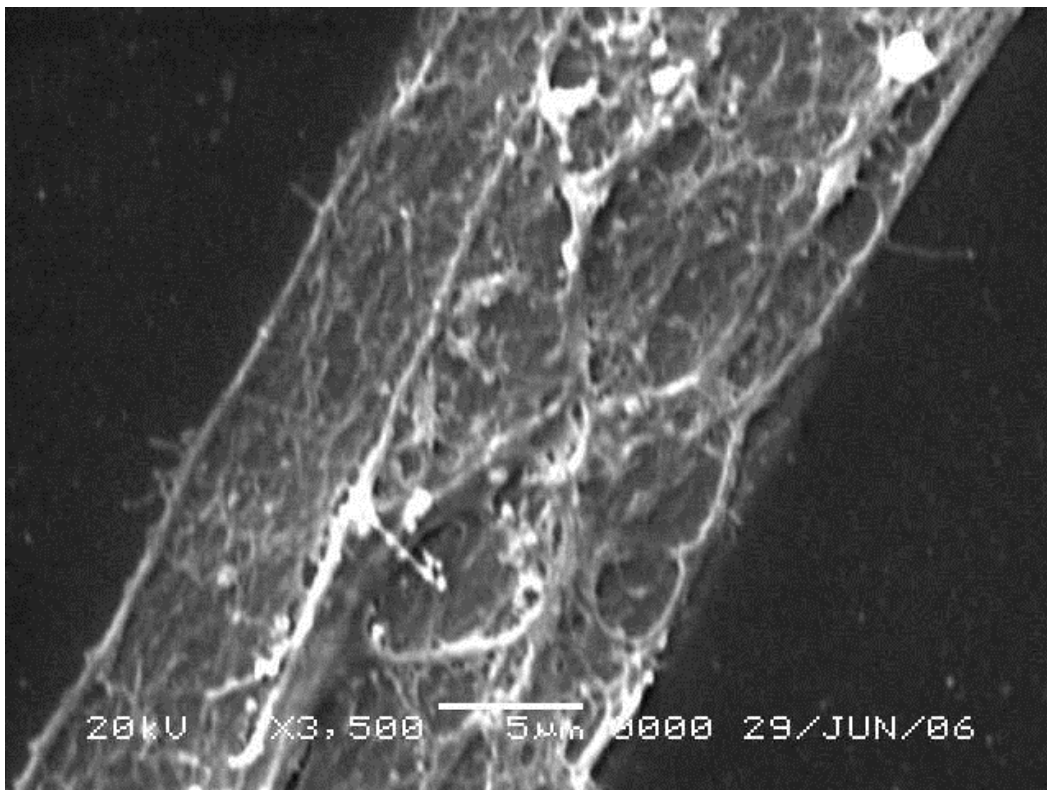
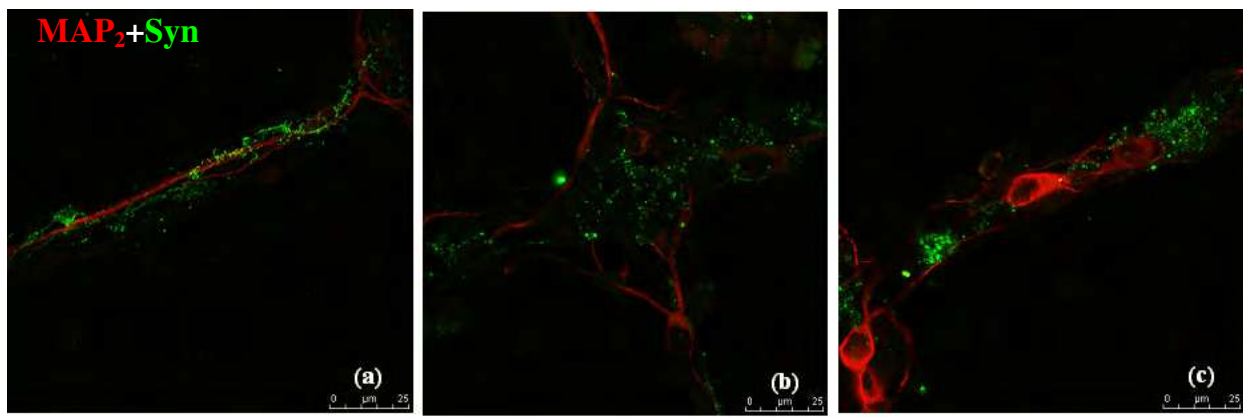


Fig. 16. SEM show that Line of grid were occupied by abound neurites.

4.2.1.2 Examination of synaptic formations by immunocytochemical staining and TEM

To further understand functional activities of neurons on the grid pattern, we observed the microstructure of the neuronal cell body, neuritis extension and processes of the synapse formation by using laser confocal microscope after 7 days in culture. It is found that several neural cell bodies aggregated on the cross point of the grid pattern (Fig.17.b), which neurites extended along the lines clearly by cytoskeletal proteins MAP2 and Synaptophysin double immunocytochemical staining of patterned neurons on PEI(Fig.17.a). Some processes can even gather into a bundle, span the distance between two cross-points and make connection with another neuron. Synaptophysin was a kind of granular protein scattered around the cell bodies and neurites, The neurites from neuron at the cross-point seemed to communicate with those at the lines which express a large number of synaptic vesicle protein(Fig.17.c).



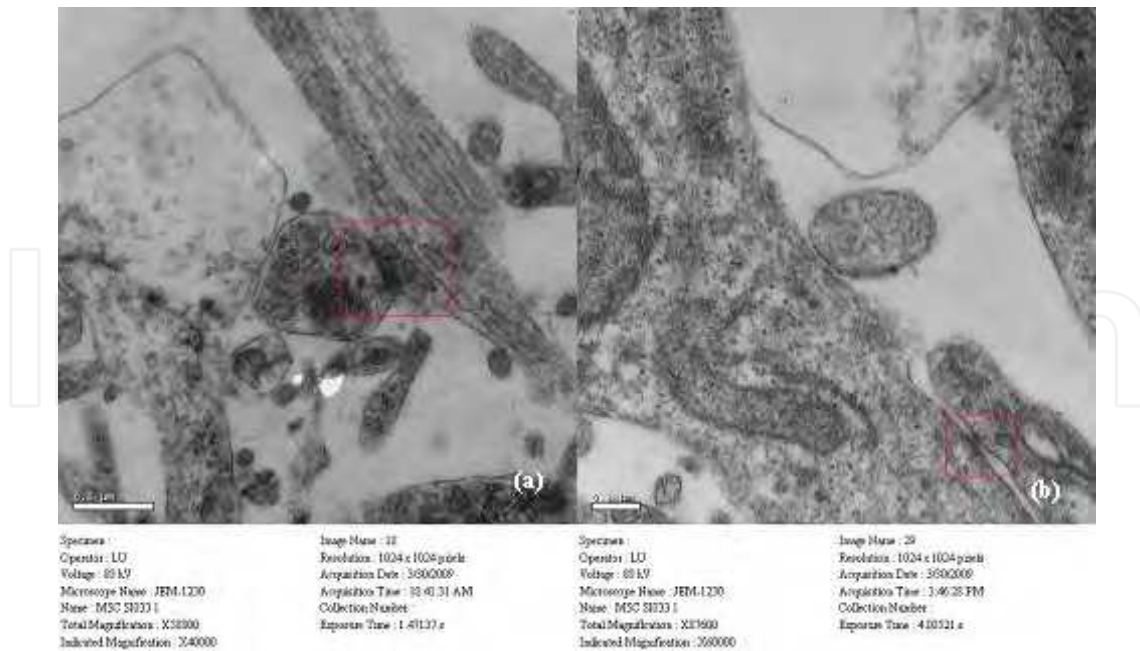
(a) neurites extended along the lines and synaptic vesicle protein around
 (b) neural cell bodies at the cross-point and synaptic vesicle protein scattering around them
 (c) synapse connection between two neurons at the grid pattern bar=25 μ m

Fig. 17. Immunofluorescent image of anti-MAP2(red fluorescence) labeled neurons and anti-synaptophysin(green fluorescence) labeled synaptic vesicle protein cultured for 7 days on PEI patterns x400

We chose PEI group which can construct more clearly neural network and continued to observe intercellular ultrastructure under transmission electron microscope (Figure18). Two periphery of neurites thicken show high electron density to form a synaptic contact (Red border). Width of synaptic cleft was measured to 30 ~ 50nm. Figure18(a) shows clear synaptic vesicles. Figure18(b) shows the synaptic cleft is relatively narrow and suspected to be electrical synapse structure due to double-membrane structure adjacent closely.

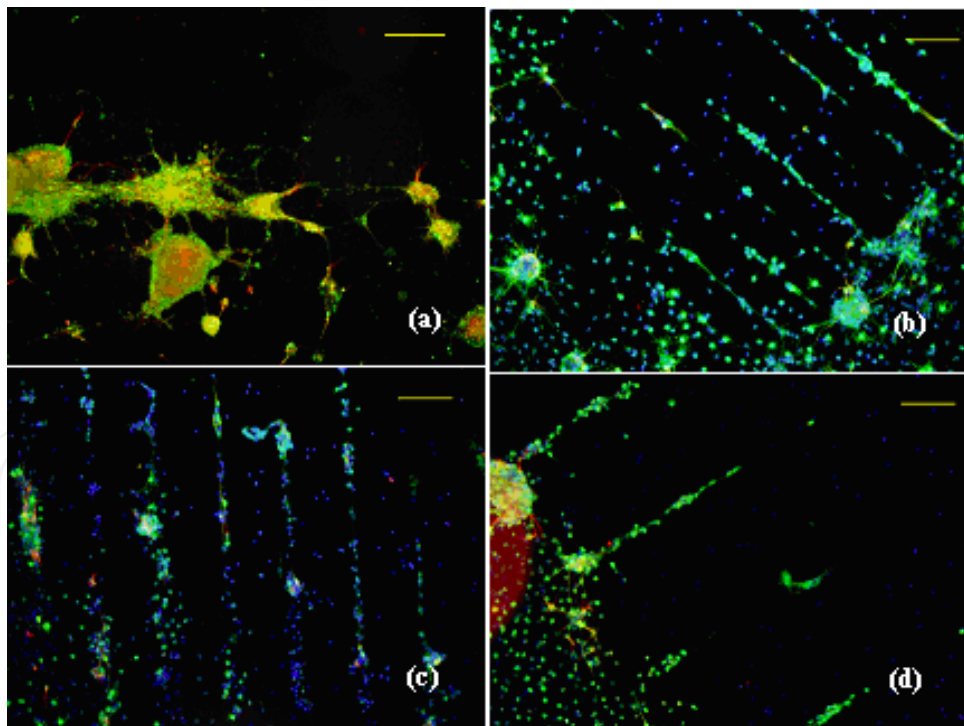
4.2.2 Neural network by microfluidic technique

In this experiment,, we made a big progress on microfluidic technique by re-designing the parameters and enhancing the photoresist coating thickness of the Cr template. After 7 days in culture, poly-l-lysine and laminin+polyethyleneimine were found to be formed more complete and clearer flow patterns by the application of microfluidic technique. On LN group, neurons were easy to cluster into clumps when channel width was 150 μ m,almost overshadowed flow pattern itself; On PEI group, even though the flow patterns are more complete, the neurites extend short and cannot constitute a connection between some cells



(a)chemical synapse structure $\times 40000$ bar= $0.5 \mu\text{m}$ (b)electrical synapse structure $\times 60000$ bar= $0.2 \mu\text{m}$

Fig. 18. Under TEM, synapse like structure between neurons from the striatum and the substantia nigra on PEI patterns

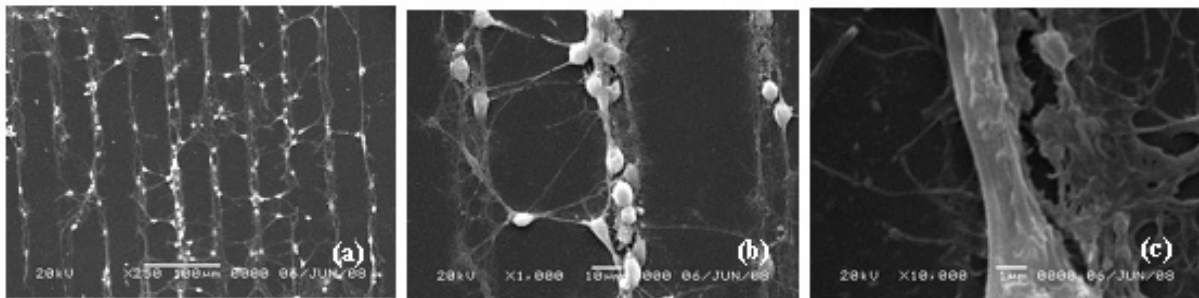


(a) LN group, width of lines $150 \mu\text{m}$, (b) PLL group, width of lines $150 \mu\text{m}$ (c) PEI group, width of lines $200 \mu\text{m}$, (d) LN+PEI group, width of lines $300 \mu\text{m}$ bar= $200 \mu\text{m}$

Fig. 19. Immunofluorescent image of anti-GABA (green fluorescence) +anti-MAP2 (red fluorescence) labelled striatal neurons cultured for 7 days on different substrates by microfluidic technique $\times 100$

sufficiently. When the channel spacing of $300\mu\text{m}$, LN + PEI group of cells in the liquid injection port at the distribution, flow pattern clear, but the group off between individual cells, so that flow interruption. Identified with immunocytochemical staining, we found that most of the MAP-2 positive neural cells from the striatum cultured on the flow pattern were also labeled with GABA, as shown in Figure 19.

Observing the fine structure of the intercellular on PLL group under scanning electron microscopic in Fig. 20, most of the neural cell bodies were found adhere to the flow pattern and majority of neurites are constrained within the width of the channel to grow following the orientation of flow channels. It is still visible that a few neuronal cell bodies deviate from the flow patterns slightly or adhere on the blank between two lines by local amplification. By observing the cross-linked region off low channel, we can see two endings of neurites seem to have varicose, swelling structure liking synapse.



(a) $\times 250$ bar= $100\mu\text{m}$ (b) $\times 1000$ bar= $10\mu\text{m}$ (c) $\times 10000$ bar= $1\mu\text{m}$

Fig. 20. Neurons from the striatum and the substantia nigra on PLL microfluidic patterns under SEM.

5. Conclusion

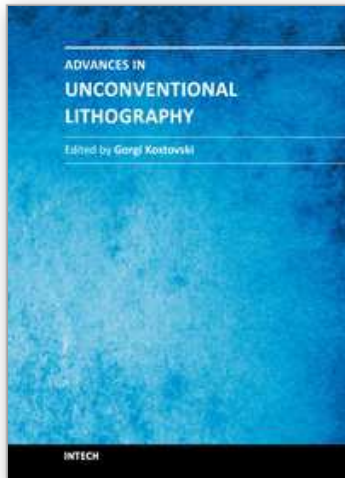
In summary, this study based on previous work improve the microfluidic technique, evaluate the influence of two different soft lithography, the micro-contact printing and microfluidic technique on various interface materials for the construction of neural network. Our future work can be divided into two levels. On the one hand continue to look for the intersection of microelectrode array and the micro-fabrication technology, trying to make cell grow in accordance with patterns of MEA electrode nodes. On the other hand, need to further improve the characteristics of electrode materials, enhance biocompatibility under the premise of improving signal-to-noise ratio and without adding resistance, do good to the survival of neurons and neurite extension.

The application of the micro-fabrication on microelectrode array may open up a broader platform of the technique for neurochip research and provide new ideas for the treatment of various injuries in the central nervous system. Ultimately, the combination both can achieve position fixing between neurons and electrodes precisely, to make the system as a real sensor, be able to accept electrical stimulation or chemical stimulation and record their signals, to analysis the transfer process of neural network information, and apply to drug screening of related diseases.

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The term Lithography encompasses a range of contemporary technologies for micro and nano scale fabrication. Originally driven by the evolution of the semiconductor industry, lithography has grown from its optical origins to demonstrate increasingly fine resolution and to permeate fields as diverse as photonics and biology. Today, greater flexibility and affordability are demanded from lithography more than ever before. Diverse needs across many disciplines have produced a multitude of innovative new lithography techniques. This book, which is the final instalment in a series of three, provides a compelling overview of some of the recent advances in lithography, as recounted by the researchers themselves. Topics discussed include nanoimprinting for plasmonic biosensing, soft lithography for neurobiology and stem cell differentiation, colloidal substrates for two-tier self-assembled nanostructures, tuneable diffractive elements using photochromic polymers, and extreme-UV lithography.

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