



Minireview

Neurons-on-a-Chip: *In Vitro* NeuroToolsNari Hong¹ and Yoonkey Nam^{2,3,*}

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Neurons-on-a-Chip technology has been developed to provide diverse *in vitro* neuro-tools to study neuritogenesis, synaptogenesis, axon guidance, and network dynamics. The two core enabling technologies are soft-lithography and microelectrode array technology. Soft lithography technology made it possible to fabricate microstamps and microfluidic channel devices with a simple replica molding method in a biological laboratory and innovatively reduced the turn-around time from assay design to chip fabrication, facilitating various experimental designs. To control nerve cell behaviors at the single cell level via chemical cues, surface biofunctionalization methods and micropatterning techniques were developed. Microelectrode chip technology, which provides a functional readout by measuring the electrophysiological signals from individual neurons, has become a popular platform to investigate neural information processing in networks. Due to these key advances, it is possible to study the relationship between the network structure and functions, and they have opened a new era of neurobiology and will become standard tools in the near future.

Keywords: axon guidance, cell culture, microelectrode array, network analysis, neural circuits, soft-lithography

INTRODUCTION

The brain is a large complex network. One of the key ques-

tions in neuroscience research has been how the complicated functions of the brain emerge from the intrinsic structures of the neural networks. To understand the underlying mechanism of the brain, dissociated neuronal cultures have been widely used as an *in vitro* model. Neurons, grown on conventional cell culture dishes, can extend processes, dendrites and axons for target selection and express ion channels for action potential generation. Moreover, they can form functional synapses between themselves, making it possible to investigate not only individual cells but also neuronal networks. Above all, *in vitro* networks have the advantages of better accessibility and easier manipulation compared with *in vivo* conditions (Feldt et al., 2011).

To establish neuronal networks *in vitro*, neurons from dissociated tissues adhere onto a culture substrate and their processes begin to grow and connect with each other. At this time, however, the positions and connections are randomly determined. This randomness makes the organized networks of dissociated neurons vastly different from the well-ordered neural networks in the brain and leads to a lack of reproducibility as an experimental model. To deal with this limitation, various cell patterning methods have been developed based on microfabrication technologies. These techniques have enabled the control of the network structures and complemented the random organization. It has also facilitated a study on the relationship between structure and function by measuring and analyzing the electrophysiological activity of the network whose structure formed to have a desired design (Hasan and Berdichevsky, 2016).

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CELL PATTERNING TECHNIQUES

Various cell patterning techniques have been used to build engineered *in vitro* networks with controlled structures by giving chemical or physical constraints in neuronal adhesion and outgrowth.

For chemical approaches, neuron-adhesive and non-adhesive regions were defined by using cell-attractive or repulsive materials (Fig. 1). Inspired by neural development *in vivo*, extracellular matrix (ECM) proteins such as laminin (Dertinger et al., 2002; Millet et al., 2010), netrin-1 (Ricoult et al., 2012), and fibronectin (Feinerman et al., 2008) were used to create the adhesive sites for the positioning and wiring of neurons. Cationic polyelectrolytes including polylysine (Chang et al., 2001; Shein-Idelson et al., 2016; Suzuki et al., 2013) or self-assembled monolayers (e.g., alkylsilane diethylenetriamine [DETA]) (Kleinfeld et al., 1988; Nam et al., 2004; Natarajan et al., 2013; Stenger et al., 1998) were utilized for cell attachment by immobilizing the glycoproteins of neurons. A mixture of proteins and polyelectrolytes were also used: laminin/PLL (poly-L-lysine) (Albers and Offenhausser, 2016; Fricke et al., 2011; Jang and Nam, 2012; Lantoine et al., 2016) and

ECM gel/PDL (poly-D-lysine) (Vogt et al., 2005; Yamamoto et al., 2018). On the other hand, non-adhesive sites were formed by cell-repulsive molecules, such as polyethylene glycol (PEG) (Edwards et al., 2013) or agarose hydrogel (Kang et al., 2009).

Based on these materials, microfabrication techniques have been applied to design spatially-distributed chemical cues on a culture substrate for designating the area of neuronal attachment. In the early stage of the technology, photolithographic patterning methods were applied to cell culture plates (e.g., glass coverslips) to generate micrometer-scale surface chemical patterns. These techniques include photolithographic etching (Corey et al., 1991; Slavik et al., 2021; Suzuki et al., 2013), photocrosslinking (Baek et al., 2011) and the lift-off process (Chang et al., 2001; Kleinfeld et al., 1988). Using photolithography subcellular resolution ($<10\ \mu\text{m}$) can be readily achieved. With the introduction of soft-lithography that utilizes replica molding of a silicone elastomer, the surface micropatterning and printing technique became more popular and versatile. It was mainly due to the fact that replica molding could be performed in a plain biological laboratory environment, and micrometer scale patterns

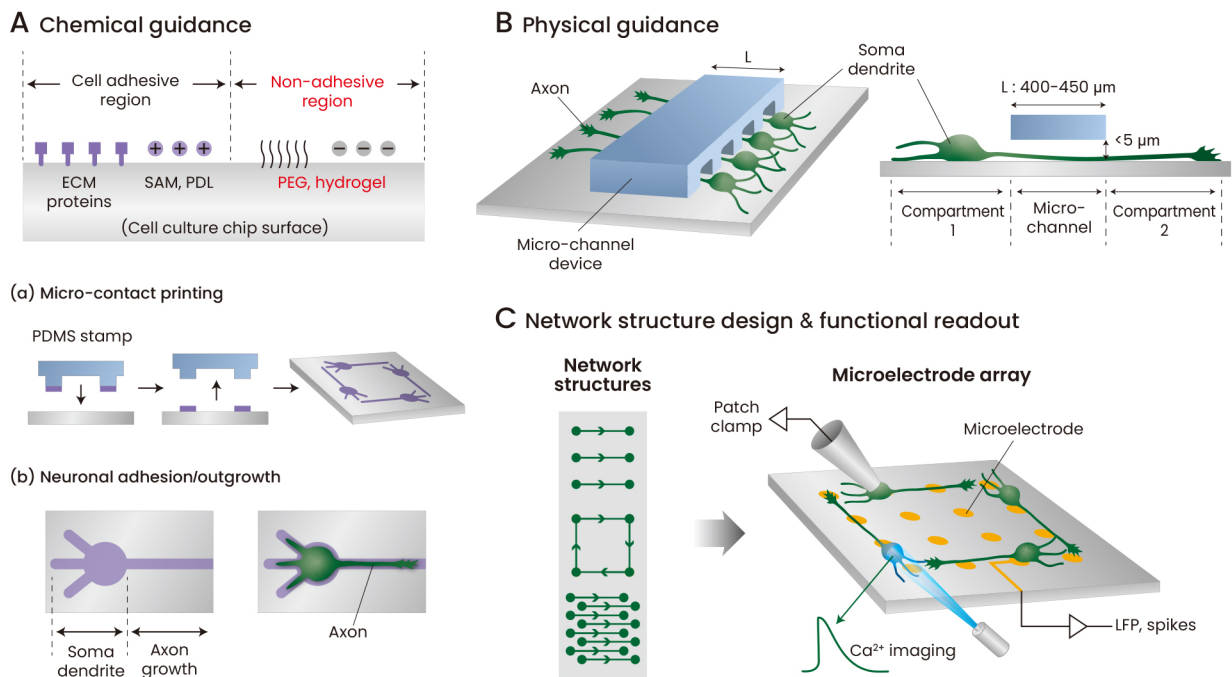


Fig. 1. Neuronal network chip design and analysis. (A) Chemical approach in the guidance of axonal outgrowth. Cell-adhesive area is defined by printing extracellular matrix (ECM) proteins, self-assembled monolayers (SAMs), and poly-D-lysine (PDL) onto the chip surface. Non-adhesive areas can be made by coating the surfaces with PEG or hydrogels (e.g., agarose). (a) Micro-contact printing scheme. A silicon elastomer (polydimethylsiloxane, PDMS) is used to fabricate a microstamp that is engraved with micropatterns. The microstamp is inked with biomolecules (purple), and the inks are transferred to the surface by contact printing. (b) An example pattern that is designed to induce axonal growth in the designated direction and locate soma. (B) The physical guidance of axonal outgrowth using a microfluidic multi-compartment device (microchannel device). Due to the height and length constraints, only axons can extend to the other compartment, thus achieving designated axonal guidance. (C) Designed network structures can be achieved on a microelectrode array that can record multiple neurons at the same time. Patch clamp recording and functional optical imaging can also be integrated to interrogate the ordered networks. LFP, local field potential.

could be readily produced with high reproducibility (Fruncillo et al., 2021; Qin et al., 2010; Whitesides et al., 2001). Using the replica molding process, one could make micro-stamps, microfluidic channel devices, and microstencils, which can be used to print or deposit desired biomolecules on a wanted area of the chip. In particular, microcontact printing uses a micropatterned elastomer stamp to transfer biomolecules to the surface of a substrate (Fig. 1). By this method, dot array patterns were generated to assess axonal collateral branching (Kim et al., 2014), synapse concentration (Ryu et al., 2016), and neuron-glia interaction (Ricoult et al., 2012). Line and grid patterns were also fabricated to investigate cell-to-cell plasticity (Vogt et al., 2005), neuronal migration and synapse formation (Lantoine et al., 2016), and differentiation and migration of neural stem cells (Joo et al., 2015). It also enabled multiple stamping with different types of biomolecules. Micropatterns with different combinations of proteins were achieved to study neuronal polarization (Shi et al., 2007) and neural stem cell differentiation (Wang et al., 2014). In addition, microfluidic devices and microstencils were used to produce chemical patterns for a stripe assay (Liu et al., 2013; Shelly et al., 2010) and network design (Shein-Idelson et al., 2016), respectively. Cell-repulsive hydrogels can be patterned on the surface by micro-molding in capillaries (Joo et al., 2018; Kang et al., 2009). This technique uses a polydimethylsiloxane (PDMS) microstamp to deliver hydrogels to a specific area. These soft-lithography based techniques showed that neurons, which were seeded on patterned cell culture substrates, followed the surface printed patterns and eventually formed networks whose form resembled the surface patterns. Some studies tried to monitor neural activity and the network structure using electrical recording techniques (Edwards et al., 2013; Jungblut et al., 2009; Ricoult et al., 2012; Yamamoto et al., 2018).

Physical patterning approaches create physical barriers on a culture substrate and control cell adhesion and neurite outgrowth. To construct physical barriers, a cell culture dish with microstructures (e.g., microwells, microgrooves, and microtunnels) were fabricated using microfabrication processes. Biocompatible polymers were used: epoxy-based photoresist (SU-8) (Berdondini et al., 2006), parylene (Erickson et al., 2008), and PDMS (Levy et al., 2012). To construct a patterned neuronal network, neurons were seeded onto the microfabricated structures, which were composed of microwells and grooves, made by photolithography (Rajnicek et al., 1997; Slavik et al., 2021) and soft-lithography (Bani-Yaghoob et al., 2005; Krumpolz et al., 2015). Additionally, several types of stencils (Hardelauf et al., 2011; Li et al., 2014) or microchannel devices (Bisio et al., 2014; Goyal and Nam, 2011) were used for physical patterning. These were used to make either single cell resolved networks or neural cluster networks. And their electrophysiological activities were measured by recording interfaces or optical imaging setups (Berdondini et al., 2006; Merz and Fromherz, 2005; Peyrin et al., 2011).

A state-of-the-art cell patterning device that acquired the most fame is the “microfluidic Campenot chamber.” The original Campenot chamber is a multi-compartmental cell culture device that can separate cell bodies and axons (Campenot,

1977). In 2005, Taylor and colleagues reported a microfluidic multi-compartment chamber that can be easily replicated by soft-lithography (Taylor et al., 2005) and installed on cell culture dishes (e.g., culture dish, glass coverslip, and recording chips). Since then, there have been various cell culture assays including axon growth (Park et al., 2014; Taylor et al., 2015), synapse manipulation (Taylor et al., 2010), synapse remodeling (Nagendran et al., 2017), axonal transport (Moutaux et al., 2018b), stem cell-derived neuron migration (Lee et al., 2014), and neuron/non-neuronal cell (e.g., glia, cardiomyocyte, and muscle fiber) interaction (Duc et al., 2021; Hosmane et al., 2010; Park et al., 2012; Takeuchi et al., 2011). More applications can be found elsewhere (Neto et al., 2016).

AXONAL GUIDANCE AND NEURONAL POLARITY CONTROL

Toward the goal of neuronal network models *in vitro*, it is imperative to control cell-to-cell connections that lead to directed neural information processing in the network. For this purpose, axonal guidance and neuronal polarity can provide the means to control the network connectivity. Both chemical and physical patterning methods were successful in regulating the axonal outgrowth with predefined directions. In chemical patterning methods, directional control of neuronal outgrowth was largely achieved in two different manners. The first is to generate a chemical gradient of proteins on the culture substrate. By using microfluidic devices, continuous gradients of laminin or laminin/PLL were produced to orient the axonal outgrowth of hippocampal neurons in the direction of increasing laminin concentration (Dertinger et al., 2002; Millet et al., 2010). Through the microcontact printing technique, discontinuous gradient patterns with a few hundreds of nanometers to a few micrometers were constructed. Laminin/PLL and ephrin5 were used to navigate single cortical neurons (Fricke et al., 2011) and retinal ganglion cells (von Philipsborn et al., 2006), respectively. By varying the gradient parameters, their effects on axonal outgrowth were assessed. The second is to define surface areas for cell body placement and neurite extension separately by pattern shapes and geometries (Edwards et al., 2013; Roth et al., 2012; Stenger et al., 1998; Yamamoto et al., 2016). The patterns of DETA, PLL, or ECM gel were composed of a cell body site (diameter: 20–35 μm) with straight or curved tracks (length: $\leq 25 \mu\text{m}$ for dendrites and $\geq 100 \mu\text{m}$ for axons, width: 1–5 μm) to position cell bodies and induce axon formation, thus achieving neuronal polarity control. These studies reported that the neurites elongated along the chemical patterns with designed neuronal polarity and neuronal signals propagated in the intended direction. Aside from these methods, there have been several studies using triangular patterns at the cellular or network scale to regulate neurite outgrowth (Jang and Nam, 2012) and activity propagation (Albers and Offenhausser, 2016; Feinerman et al., 2008).

Recently, microfluidic multi-compartment chamber devices have been the leading tool to induce unidirectional connections and activity propagation. Axon specific outgrowth can be achieved by controlling the height and length of the

channels (Taylor et al., 2005) (Fig. 1). When microchannels have a height less than 5 μm , only neurites can grow into the channels (tunnels), and large cell bodies cannot migrate into the channels. When the length is longer than 400-450 μm , only axons can extend, and dendrites are less likely to outgrow more than 400-450 μm . Based on this principle, microchannels with asymmetric shapes were designed, and controlled axonal outgrowth and signal propagation in the desired direction were achieved (Forro et al., 2018; Gladkov et al., 2017; le Feber et al., 2015; Peyrin et al., 2011; Renault et al., 2016). In addition to designing the geometry of the microchannel devices, a sequential seeding method in the culture chambers of a microchannel device was reported to establish a unidirectional connectivity (Pan et al., 2011).

IN SITU CONTROL OF NEURITE OUTGROWTH

Most of techniques for neuronal cell patterning are static in a sense that patterns are generated before cell seeding and cannot be altered thereafter. To fully control neuronal network models, pre-designed and established neural connections should be manipulated in an *in situ* manner. *In situ* cell patterning techniques could navigate neurites and establish new neuronal connections even after the cultivation, enabling precise directional control and rewiring between neurons. In addition, they could inflict damage on neurons and eliminate selective connections, making it possible to implement axonal injury and neural disease models.

Photoinduced processes have been applied to guide neurites and form connections between neurons during cell cultivation. The high spatial resolution of light-mediated approaches have enabled surface modification in a defined region near growing neurons at micrometer scales. Photochemical reaction uses a photo-reactive self-assembled monolayer (SAM) with a photocleavable group such as 2-nitrobenzyl group (Edagawa et al., 2012). This functional group could be released by localized UV (ultra-violet) irradiation, consequently peeling off a neuron-repellent layer on the SAM during the cell cultivation. The induction of new neurite elongation in the UV irradiated areas has been demonstrated with cultured PC12 cells. Photoablation uses a laser to selectively remove the surface coatings. Individual neurons were arranged on each chemical pattern that was generated with cell-attractive DETA and cell-repulsive octadecylsilane (ODS) before cell seeding (Yamamoto et al., 2011). After two days, the ODS, which was blocking the connection between neurons, was locally removed using a femtosecond laser, and laminin was adhered on the ODS-removed surfaces, so that the neurites grew along the laminin lines and connected separated neurons. It has been also shown that cell-to-cell connections could be established through selective removal of cell-repellent perfluoroalkyl polymer via the photoablation process (Okano et al., 2011). Photothermal etching uses a cell-repulsive agarose hydrogel which can be melted by heat. The heat was generated by a photo-absorber layer including indium-tin oxide ($\lambda = 1,064 \text{ nm}$) (Suzuki et al., 2004), chromium ($\lambda = 1,064 \text{ nm}$) (Hattori et al., 2004), or gold nanorods ($\lambda = 785 \text{ or } 808 \text{ nm}$) (Hong and Nam, 2020) with a focused laser beam. Depending on the laser wavelength ($\lambda = 1,480$

nm), the direct heating and melting of an agarose hydrogel were also possible (Hattori et al., 2004; Suzuki et al., 2005). Using this technique, individual neurons or neuronal populations in each chamber (ten to hundreds of micrometers in diameter or width) were connected with each other by photothermal etched microgrooves or tunnels (a few to tens of micrometers wide) during cultivation.

Apart from these photoinduced methods, electric and magnetic energies were used. By integrating a microfluidic device and gold electrodes on glass substrates, it was demonstrated that an AC (alternating current) electrokinetic force stopped the growing of neurites adjacent to the electrodes, thereby gating the neurite growth into the microchannels and creating a directional connection between neuronal clusters (Honegger et al., 2013; 2016). Magnetic fields were also applied to move a microrobot for wiring neuronal networks the day after cell seeding (Kim et al., 2020). Direct mechanical pulling was also used to elongate neurites and connect neural circuits using PDL microbeads and AFM (atomic force microscopy) tips or pipettes (Magdesian et al., 2016).

MICROELECTRODE ARRAY FOR ANALYZING THE FUNCTIONAL CONNECTIVITY OF NEURONAL NETWORKS

To study the functional features that emerge from the underlying structure of the brain, technologies for recording electrophysiological activities have been used in neuronal culture systems. Among the many neural recording interfaces, a microelectrode array (MEA), which is an array of micrometer scale electrodes, has been widely used as an electrical read-out platform (Nam and Wheeler, 2011). One great advantage of the MEA is simultaneous recording of extracellular signals (local field potentials and neural spikes) from multiple electrodes (Fig. 1). This method enables one to obtain spatiotemporal information from neuronal networks. Previous studies attempted to relate structural and functional connectivity by combining the MEA system with optical imaging techniques (Okujeni et al., 2017; Ullo et al., 2014). Moreover, because the MEA is a non-invasive interface to the cell, it enables long-term monitoring of functional development and dynamics during network formation and maturation. Based on the feature, the developmental changes in the functional connectivity of cultured networks were assessed by acquiring spontaneous activities for a few weeks (Downes et al., 2012; Schroeter et al., 2015).

A variety of neuronal patterning techniques have been applied to the MEA for several purposes. Using chemical patterning techniques, neuronal networks with controlled geometries (e.g., line, grid, and triangle) could be established on MEAs, and their electrical activities were successfully measured (Jungblut et al., 2009; Marconi et al., 2012; Nam et al., 2004; Suzuki et al., 2013). To mimic the modularity of the brain, interconnected neuronal clusters (80-200 μm in diameter) were built on individual electrodes, and signal transmission between the clustered networks was analyzed (Joo et al., 2018; Shein-Idelson et al., 2016). By making physical barriers on the MEA, coupled neuronal populations with larger scales (several mm^2) were also developed (Berdondini et al., 2006;

Bisio et al., 2014; Levy et al., 2012). Based on spontaneous recordings or electrical stimulations, activity propagations within and between modules were examined.

The integration of microfluidic multi-compartment devices, capable of separating cell bodies and axons, on MEAs made it possible to form a multi-compartment neuronal network that is connected by only neurites, particularly axons. By introducing an asymmetric design into the microchannel structure, directed connections between compartments could be assigned, and signal propagation was predominant in the defined direction (Forro et al., 2018; Gladkov et al., 2017; le Feber et al., 2015; Moutaux et al., 2018a). The integrated system of an MEA and a microchannel device facilitated a co-culture study. To investigate the sub-circuitry of the brain, neurons from different regions were seeded in each compartment. On the MEA, the DG-CA3-CA1 (Brewer et al., 2013) and cortical-thalamic network (Kanagasabapathi et al., 2012) were developed, and their spike propagation and burst behavior were examined. Furthermore, different cell types, such as cardiomyocytes with neurons in the peripheral nervous system (Takeuchi et al., 2011), myoblasts with motor neurons (Duc et al., 2021), or stem cell-derived neurons with primary neurons in the central nervous system (Takayama et al., 2012) were also co-cultured to interrogate their interactions via electrophysiological observation.

Recently, an MEA platform has been applied to study brain organoids. Different types of organoids, such as cortical organoids (Fair et al., 2020; Trujillo et al., 2019), cerebral organoids (Osaki and Ikeuchi, 2021), and engineered organoids, which consist of excitatory and inhibitory neurons with supportive glial cells (Zafeiriou et al., 2020), were placed on the microelectrodes. For several months, organoid functionality including complex oscillatory and synchronous network bursting were assessed.

PERSPECTIVES ON FUTURE NEURONS-ON-A-CHIP TECHNOLOGY

The Neurons-on-a-Chip platform has now reached the level of designing networks with cells. It is possible to create a network using various cells, design a structure by manipulating the connections between networks, and measure the change in function simultaneously in multiple cells. At the core of this, micro-technology, which has been developed in the past decades and established as a base technology in the biological field, has played a major role. In particular, the micro-electrode array platform is being developed into a large-scale screening platform to ensure fast, reproducible, and reliable readouts. Now, the Neurons-on-a-Chip will develop into a platform to design and measure a 3D neural network beyond the 2D. It will be reborn as a three-dimensional neuro-tool that manipulates and measures the physiological activities of organelles and cellular levels while mimicking the micro-environment of the body *in vitro*. Emerging nanotechnologies will be integrated with the existing platforms to extend the dimensionality.

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AUTHOR CONTRIBUTIONS

N.H. and Y.N. wrote the manuscript and prepared the figure.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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