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Synaptic compartmentalization by micropatterned masking of a surface adhesive cue in cultured neurons

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ABSTRACT

Functions of neuronal circuit are fundamentally modulated by its quality and quantity of connections. Assessment of synapse, the basic unit for a neuronal connection, is labor-intensive and time-consuming in conventional culture systems, due to the small size and the spatially random distribution. In the present study, we propose a novel 'synapse compartmentalization' culture system, in which synapses are concentrated at controlled locations. We fabricated a negative dot array pattern by coating the entire surface with poly-l-lysine (PLL) and subsequent microcontact printing of 1) substrates which mask positive charge of PLL (Fc, BSA and laminin), or 2) a chemorepulsive protein (Semaphorin 3F-Fc). By combination of physical and biological features of these repulsive substrates, functional synapses were robustly concentrated in the PLL-coated dots. This synapse compartmentalization chip can be combined with the various high-throughput assay formats based on the synaptic morphology and function. Therefore, this quantifiable and controllable dot array pattern by microcontact printing will be potential useful for bio-chip platforms for the high-density assays used in synapse-related neurobiological studies.

1. Introduction

The morphological and functional complexity of the nervous system comes from the synapse, where axons of one neuron (presynaptic cell) send signals to dendrites of another neuron (postsynaptic cell). When action potentials arrive at a chemical synapse, neurotransmitters released from presynaptic terminals bind to specialized receptors in the postsynaptic sites, leading to activation of channels or production of chemical messengers. Processing and integration of a large amount of inputs from the environments results in alteration of synaptic strength, elimination of the existing synapse or production of a new synapse. These processes are called synaptic plasticity, which is essential for learning and memory. A large number of *in vitro* systems have identified various molecules and signaling pathways essential for synapse structures and their modulation [1]. However, the study of neuronal synapse is limited by the inability to efficiently analyze the structure, number and size of the synapse because of its structural complexity and small size. Visualization of synapses at high magnification spots a tiny fraction of whole axons and dendrites, which often leads to selection bias in the analysis. Therefore, a system for positioning and concentrating synapses in an identified area, which is not possible in a regular neuronal culture system, will be more efficient and helpful in analyzing the synaptic functions, facilitating high-throughput analysis.

Microfabrication techniques, such as stripe assay, microfluidics, laser-assisted patterning and microcontact printing, have been developed to investigate neuronal growth and functions in a simple and precisely controllable way [2–4]. Microcontact printing, by which a variety of surface-bound structures can be printed, has been successfully applied to neuronal culture at subcellular scales, especially for understanding the mechanisms of axon guidance and axonal growth. Micropatterned PLL has been used for directing axon or neurite outgrowth [5,6]. A microdot array by use of PDL has been adopted to control axon branch formation [7]. Additionally, it has been shown that microcontact printing with extracellular matrix, laminin, and adhesion molecules, neuroligin and NgCAM, changed the growth pattern of neurites [8–10]. Ephrin, an axon

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guidance molecule, was used for microcontact printing to produce discontinuous gradients. Growth cones of chick retinal axons responded to ephrin gradients, forming stop zones at a distinct range [11]. So far, most of the studies have focused on axon or neurite outgrowth in short-term cultures. Microcontact printing has the advantages of providing stability over a long time [2] and spatially modulating the complicated neuronal morphology by patterned shapes [12]. Therefore, we fabricated a dot array pattern by using microcontact printing to spatially control synapse formation in long-term cultures.

In this study, we developed a novel neuronal culture system that allows the concentration of synapses at the desired position. Unlike the previous studies, we coated the surface of the coverslip with adhesive substrate, PLL, and subsequently printed the dot pattern with use of the masking/repulsive substrates on the top of PLL (substrate/PLL). Axons and dendrites preferentially extended into printing-negative dots, and synapses were focally concentrated into the dots, causing synapse compartmentation. This synapse compartmentation system offers at least three key benefits for studying the synapse. First, focalized synapses in an identified area are easily and efficiently analyzable, which enables semi-automated quantification using the analysis software. Second, the content of the synapse formed in the dot is controllable by the dot size depending on the experimental purposes. Third, the combination with other systems including an MEA makes this system extensible for examining neuronal connectivity.

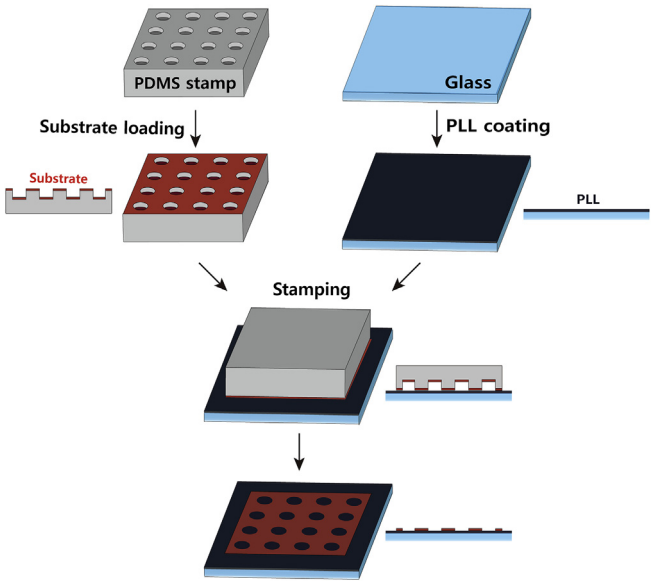

Fig. 1. Microcontact printing and micropatterned dot array chips. A procedure for microcontact printing. Substrate solution was overlaid on a PDMS stamp and the stamp was applied to a PLL-coated coverslip. The substrate was printed in the area outside the dot in the negative dot array.

2. Results

2.1. Synapse compartmentalization in the negative dot array

Spontaneous synapse formation involves axons and dendrites of adjacent neurons in a conventional neuronal culture system. As axons and dendrites are randomly located *in vitro*, synapses are hence randomly distributed in a culture system. We hypothesized that if the location of axons or dendrites is spatially controlled, synapse formation can be spatially directed into the desired location with pattern. Sema3F is one of the well-known chemorepulsive molecules, which acts as an axon guidance molecule and also affects dendritic growth through binding to its receptor, neuropilin 2 (Nrp2) [13–17]. We subjected Sema3F to microcontact printing to investigate whether synapse formation can be spatially confined by a patterned substrate printing. Sema3F-Fc chimera (10 μg/ml) and Fc control (10 μg/ml) were employed as a printing substrate for the negative dot array, which contains an array of PLL-coated dots surrounded by a substrate printed on PLL (Fig. 1). Microcontact printing with Sema3F-Fc and Fc was confirmed by staining the printed coverslip with an anti-Fc antibody after stamping (data not shown). In the negative dot array pattern of 100 μm in diameter and 100 μm in spacing (100D-100S), printed with Fc and Sema3F on PLL, distribution of presynaptic and postsynaptic compartments was visualized with synapsin and PSD95, respectively. Surprisingly, synapses were focally concentrated inside the dots (PLL only) in a 3-week-old culture in both groups (Fig. 2A). We performed most of experiments after 21 days *in vitro* (DIV) as clustering of mature synapses was clearly seen from 21 DIV onwards. For precise quantification of synapse enrichment in the negative dot, we obtained two ratios as shown in the diagram of Fig. 2B. First, we measured the density of synaptic components inside the dots (*in*) and in the adjacent area (*out*) (Fig. 2B, top) and obtained the ratio of these two values. This ratio (*in/out*) was significantly higher in Sema3F than in Fc control, for both presynapses and postsynapses (Fig. 2B). Second, the fluorescence intensity in the dot (*on*) was compared to that in the substrate printing area with the same dot size spatially separated from the dot (*off*) (Fig. 2B, bottom). The ratio (*on/off*) demonstrated that both presynapses and postsynapses concentrate more into the dot in the negative dot array printed by Sema3F than by Fc control. However, Fc control itself generated the pattern of synapse clustering (Fig. 2A and B). These two ratios (in/out and on/off) would be 1 if synapse formation had no preference for the location indicated by the theoretical line in Fig. 2. In random cultures homogeneously coated with PLL, the ratio (*in/out*) was close to 1 (Fig. 2D). The result from Fc control raised the possibility that macromolecules may trigger synapse clustering by masking adhesive characteristics of the PLL-coated surface. We examined whether BSA (1 mg/ml) and laminin (10 μg/ml) printing also produce synapse compartmentalization in the negative dot array. BSA (molecular weight 66.5 kd) is commonly used as a blocking reagent and is easily available in the biology laboratory. Laminin, a high molecular weight (400 kd) protein extracellular matrix, is another common protein utilized for cell adhesion and migration. Compared with random culture on PLL coated coverslip without a pattern, both substrates exhibited strong synapse compartmentalization (Fig. 2C and D), indicating that microcontact printing of various substrates can be used for spatial patterning of the synapses.

2.2. Physical and functional properties of substrates controlling synapse location in the negative dot array

To gain insight into the mechanism by which substrate printing directed synapse enrichment into the PLL-coated area, we first examined the physical properties of substrates. PLL is generally used as a coating material for primary neuronal culture, as its positive charge enhances cell attachment and adhesion to plastic ware and glass coverslips. Therefore, we hypothesized that the surface potential difference between negative dots (PLL only) and surrounding printing areas (substrate on PLL) induced synapse compartmentalization into the negative dot. To investigate the effect of surface charge on synapse compartmentalization, we prepared samples with 10 μm patterns of printed substrate on PLL background. Then, topography, mechanical property and surface potential were observed by using an atomic force microscopy