Unidirectional signal propagation in primary neurons micropatterned at a single-cell resolution

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The structure and connectivity of cultured neuronal networks can be controlled by using micropatterned surfaces. Here, we demonstrate that the direction of signal propagation can be precisely controlled at a single-cell resolution by growing primary neurons on micropatterns. To achieve this, we first examined the process by which axons develop and how synapses form in micropatterned primary neurons using immunocytochemistry. By aligning asymmetric micropatterns with a marginal gap, it was possible to pattern primary neurons with a directed polarization axis at the single-cell level. We then examined how synapses develop on micropatterned hippocampal neurons. Three types of micropatterns with different numbers of short paths for dendrite growth were compared. A normal development in synapse density was observed when micropatterns with three or more short paths were used. Finally, we performed double patch clamp recordings on micropatterned neurons to confirm that these synapses are indeed functional, and that the neuronal signal is transmitted unidirectionally in the intended orientation. This work provides a practical guideline for patterning single neurons to design functional neuronal networks *in vitro* with the direction of signal propagation being controlled. *Published by AIP Publishing.* [<http://dx.doi.org/10.1063/1.4959836>]

Advances in biointerface engineering technologies over the last two decades have made it possible to reconstruct neuronal circuits from once-dissociated neurons. This has provided neuroscience with a novel system to study the development and emergent functions of living neuronal networks.^{1,2} In neuronal networks, signal transmission between neurons occurs at specialized contact sites called synapses. Primary neurons in dissociated cultures are capable of forming functional synapses,^{3,4} even when they are grown on the engineered surfaces.^{5–11} Boosted by the contemporaneous development of microelectrode-array technology, the surface patterning technique has been utilized to investigate how the structure of a network affects spatiotemporal patterns of synapse-dependent neuronal activity.^{12–16}

The patterning of cultured neurons is often conducted at a multicellular scale due to the technical ease of both pattern fabrication and cell culturing. Single-cell patterning is nevertheless important, since it allows for the simultaneous control of the parameters that define a network structure, such as axon-dendrite polarity^{2,17–20} and cell types.²¹ Previous studies have shown that the membrane properties of primary neurons grown on patterned substrates are similar to those grown on conventional unpatterned substrates⁶ and are capable of

forming functional synapses that are equivalent to those of unpatterned neurons.^{7,8}

In this letter, we report on a single-cell patterning strategy to design neuronal circuits with predefined signal propagation direction. Using micropatterns that direct axons in a predefined orientation, we show that synapses are formed on dendrites and soma of the micropatterned neurons after a sufficient period of culturing. The density of synapses on micropatterned neurons was found to depend on the geometry of the micropattern. Finally, we demonstrate that these synapses are indeed functional and that the signal propagates unidirectionally as it was designed to.

Microcontact printing (μ CP) was used to pattern a mixture of poly-lysine/extracellular matrix gel (PECM) on an octadecylsilane-coated coverslip [Fig. 1(a)]. Micropatterns consisted of a circular island for a soma (35 μ m diameter), a long path for an axon (100 μ m), and short paths for dendrites (20 μ m).¹⁹ Individual patterns were serially aligned with 10 μ m gaps. Three pattern geometries were compared: bipolar, quadrupolar, and octupolar patterns comprised one, three, and seven short paths, respectively. The quadrupolar pattern was our starting point, since it is known to be capable of directing axon-dendrite polarity.¹⁹ The bipolar pattern is a geometry that is also frequently used in neuron patterning experiments and has been employed in a number of polarity control experiments. The octupolar pattern was intended to have "more than enough" paths for dendrites because a hippocampal neuron develops an average of 4.9 dendrites.²²

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FIG. 1. Fabrication of the micropatterned substrates. (a) Schematic illustration of the process. Cell permissive proteins were patterned on a coverslip by μ CP. Rat hippocampal (HC) neurons were attached to the coverslip, and then the coverslip was placed upside-down to face the astrocyte feeder layer which was grown in a separate dish. (b) Fluorescence micrographs of the protein pattern, visualized by adding a fluorescent dye (SR101) to the protein solution. Scale bar, 100 μ m.

The proper transfer of the PECM ink was confirmed by visualizing the pattern geometry with fluorescence microscopy using protein ink that contained $10 \mu\text{g ml}^{-1}$ of sulforhodamine 101 (SR101) [Fig. 1(b)]. When growing primary neurons, neurons were first plated on a coverslip, which, after 3 h incubation, was flipped upside-down and transferred to a dish with a glia feeder layer [Fig. 1(a)]. See supplementary materials for details.²³

First, to verify that the PECM ink supports the proper growth of primary neurons and the formation of synaptic contacts, rat hippocampal neurons were grown on a glass coverslip that had been uniformly coated with PECM. Signatures of properly growing hippocampal neurons, such as well-spread soma, growth of thick proximal dendrites, and un-bundled axons, were confirmed from phase-contrast images [Fig. S1(a)²³]. Moreover, synaptic contacts on

dendrites and soma could be observed at 10 days *in vitro* (DIV) as colocalized puncta of presynaptic and postsynaptic markers Syn1 and PSD95, respectively [Figs. S1(b) and S1(c)²³]. These results validate the use of PECM as ink for μ CP.

Next, we studied the orientation of axon-dendrite polarity on the micropatterns. We previously investigated neuronal polarization on an isolated quadrupolar pattern and showed that the neurite that grows on the long path is fated to be its axon, and that the others become dendrites.¹⁹ Immunostaining for axonal and somato-dendritic markers tau-1 and MAP2, respectively, revealed that neurons were polarized in the intended orientation for all pattern geometries and that the polarity control was still effective even after the patterns were aligned with minor gaps [Fig. 2(a)]. Quantification of the percentage of axons growing toward the long path versus the opposite direction revealed no difference among the three patterns [Fig. 2(b); $n > 74$ cells]. Soon after the formation of an axon at 1–2 DIV, neurites were frequently observed to cross over the 10 μ m gap, a phenomenon that has been reported by other investigators.²⁴ The observation that axons preferentially orient in the direction of the longest path shows that a 10 μ m gap is an effective barrier for confining minor processes of premature neurons. It also suggests that axons have a greater capability of crossing the gap than minor processes. Phase-contrast observations of single hippocampal neurons growing on the micropattern at 1 and 7 DIV show that the fidelity of the pattern geometry is retained throughout the culture durations required for synapse development, and that axons that crossed the 10 μ m gap physically contacted the targeted neuron [Fig. 2(c)].

We then studied whether these physical contacts led to the formation of synapses. Cultured hippocampal neurons start to form synapses at around 4 DIV, which grow in number as the cultures mature.⁴ Fig. 3 shows single neurons on quadrupolar micropatterns immunostained for synaptic markers at 6 and 10 DIV. At 6 DIV, some small Syn1-positive puncta were observed but these puncta were frequently not colocalized with the postsynaptic marker PSD95 [Figs. 3(a) and 3(b)]. The puncta most probably correspond

FIG. 2. Polarization of hippocampal neurons on micropatterns. (a) Fluorescence micrographs of 2 DIV neurons on the three patterns stained with an axonal marker (tau-1; red) and a somatodendritic marker (MAP2; green). Scale bar, 20 μ m. (b) Percentage of compliant axons, i.e., axons growing on the longest path, for the three patterns. (c) Phase-contrast micrographs of neurons growing on the quadrupolar micropatterns. Axons are oriented towards the right. After 1–2 days in culture, axons cross over the minor gap (10 μ m) to make physical contacts with the neighboring neuron. The cells were observed at 1 DIV (middle panel) and 7 DIV (right panel).

FIG. 3. Distribution of synapses on micropatterned neurons (MAP2, grey; Syn1, green; PSD95, red). Neurons were grown on quadrupolar micropatterns with the axon orienting upwards, as indicated with arrows in panels (a) and (c). (a) At 6 DIV, most of the Syn1-positive puncta are small, and PSD95-positive puncta are rarely observed. For this specific neuron, no synapses were found. Scale bar, 20 μm . (b) Enlarged view of the boxed region in panel (a). Syn1-positive puncta are observed, but they are small, sparse, and not colocalized with PSD95 puncta. Scale bar, 2 μm . (c) By 10 DIV, many of the Syn1-positive puncta have larger size and are colocalized with PSD95. (d) Enlarged view of the boxed region in panel (c). Syn1-positive puncta observed at 6 DIV are dimmer, and hence brightness levels of Syn1 were adjusted accordingly to aid visualization of its localization.

to the clusters of synaptic vesicles undergoing axonal transport.³ The cultures further matured by 10 DIV, and Syn1 puncta were observed to be enlarged and colocalized with PSD95 [Figs. 3(c) and 3(d)]. Colocalization was observed on both dendrites and soma, similar to conventional, homogeneous cultures. Quite interestingly, synapses were also formed at the axon initial segment region [Fig. 3(c)], but it should be noted that axo-axonic synapses were also observed in other cells.

Quantification of synapse densities on dendrites and soma during development is shown in Fig. 4. The density of synapses of neurons grown on a homogeneous PECM surface increased as the cultures matured. Similarly, synapse density increased with culture time for neurons grown on quadrupolar and octupolar patterns. In contrast, no increase in synapse density was observed at 10 DIV for neurons on bipolar patterns. Note that a single presynaptic axon can form multiple synapses along its length, termed the *en passant* synapses.^{25,26} Statistical analyses revealed that there was no significant difference between quadrupolar and

FIG. 4. Quantitative analysis of synapse density on (a) dendrites and (b) soma. No statistically significant difference was found between the numbers for quadrupolar and octupolar patterns throughout the culture time. ** $p < 0.01$ against quadrupolar and octupolar. Error bars represent s.e.m. Note that the synapse density in the homogeneous (unpatterned) and patterned cultures cannot be compared directly, since it depends strongly on the probability of axons encountering postsynaptic neurons and on the pathway of axon elongation after the contact.

octupolar patterns throughout the experiment. Moreover, synapse density at 10 DIV was significantly lower on the bipolar pattern compared to the other two pattern geometries ($p < 0.01$). This observation indicates that growing neurons on the bipolar pattern affects the maturation phase of synapse formation.

Finally, we confirmed that synapses formed by micropatterned neurons are physiologically functional. Fig. 5 shows representative traces from a double patch-clamp recording of two neighboring neurons aligned on the octupolar pattern. Stimulation of the "upstream" neuron (cell 1 in Fig. 5) caused the cell to fire an action potential, which then propagated and generated excitatory postsynaptic potential (EPSP) in the "downstream" neuron (cell 2) [Fig. 5(a)]. In contrast, no EPSP was observed in cell 1 by the stimulation of cell 2 [Fig. 5(b)]. This shows that synapses between these neurons are physiologically functional and that the signal propagates unidirectionally in accordance to the micropattern geometry. The distribution of EPSP amplitudes is shown in Fig. 5(c). For each neuron pair, we evaluated average EPSP amplitudes over multiple stimulations. The median over different neuron pairs was 2.6 mV, 5.3 mV, and 3.8 mV for the bipolar ($n = 13$ pairs), quadrupolar ($n = 10$ pairs), and octupolar patterns ($n = 6$ pairs), respectively. The EPSP was

FIG. 5. Double patch-clamp recordings from micropatterned neurons. (a) The stimulation of cell 1 generated an action potential in cell 1, and an EPSP was observed in cell 2. (b) The stimulation of cell 2 generated an action potential in cell 2, but in this case, an EPSP was not observed in cell 1. The traces presented are from the octupolar pattern at 10 DIV. (c) Distribution of EPSP amplitudes for the three micropattern geometries recorded at 10–11 DIV: bipolar (green), quadrupolar (blue), and octupolar (red).

blocked in the presence of either a sodium channel blocker, tetrodotoxin ($1\ \mu\text{M}$), or an AMPA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione ($10\ \mu\text{M}$), and was recovered after washout (data not shown). Signal propagation was observed in 57% of the neuron pairs, in which 77% were connected unidirectionally. Cultured neurons are usually connected bidirectionally on a homogeneous substrate. The present result shows that micropatterns can be used to effectively control their connectivity at a single-cell resolution.

Hippocampal neurons begin to form synapses after 3–4 DIV, and the rate limiting step in this process is the maturation of dendritic processes.⁴ This led us to hypothesize that the dendritic geometry of micropatterned neurons might affect the efficiency of their synapse formation. Interestingly, no significant difference in synapse development was observed between the quadrupolar and octupolar patterns throughout the period of observation. This suggests that bearing three dendrites is sufficient for a hippocampal neuron to mature normally and present appropriate postsynaptic specializations. On the other hand, synapses that were formed on neurons on the bipolar pattern were sparser compared to those on the other two patterns (Fig. 4). We interpret this as a negative side effect associated with excessive cell confinement. Rat hippocampal neurons grow multiple dendrites in the absence of geometrical confinement. However, the cells in the present study were able to grow only one dendrite on the bipolar pattern. Previous studies on endothelial cells have shown that the inhibition of cell spreading alters cell function and even triggers cell death.^{27,28} In support of this, cell bodies of neurons on the bipolar patterns often appeared to be swollen by 10 DIV, as evidenced by phase-contrast microscopy. Although the exact molecular mechanism has not been fully clarified, Rho-GTPase signalling is suggested to be involved.²⁹

The finding that axons cross the minor gap between micropatterns and yet the neurons polarize according to the micropattern is an important step in engineering neuronal circuits. However, the alignment of the micropatterns decreased the fidelity of polarity guidance, leading to $\sim 30\%$ of the axons to grow “backwards.” The growth of axons in the wrong direction was not observed when each pattern was isolated, with a separation of more than $100\ \mu\text{m}$.¹⁹ Extension of the gap should increase the chances of having axons on the

longest path, and this would be effective for constructing neuronal circuits with defined orientation of signal propagation. Furthermore, the employment of *in-situ* neurite guidance techniques that allow post-processing of the adhesive micropatterns^{9,30–32} would further increase the efficiency of controlling circuit structures.

In conclusion, we investigated the development of synapses in rat hippocampal neurons grown on micropatterned surfaces. We found that synapses start to develop at approximately the same age as they do in a homogenous culture. On micropatterns that permit the growth of multiple neurites, synapse density increased with culture age. In contrast, when only a single dendrite was permitted to grow, the increase ceased irregularly at 10 DIV. Using a micropattern with multiple dendrites, we further showed that synapses formed between micropatterned neurons are functional and that the signal transmission occurs unidirectionally. The induction of synapse formation is a critical step in constructing neuronal networks with a predetermined connectivity, and the results reported herein provide important guidelines for designing the micropatterns.

Interests in the analysis of a biological system at a single-cell level are growing not only in neuroscience but also in other areas such as mechanobiology,^{33–36} stem cell biology,^{33–35} clinical diagnostics,^{34,36} and drug discovery.^{37,38} State-of-the-art micro/nano-technology, including surface engineering and microfluidics, is the prime factor driving this trend. Further technological improvements will clearly open a new field in biology, medicine, and biophysics.

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