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Noninvasive Neuron Pinning with Nanopillar Arrays

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ABSTRACT Cell migration in a cultured neuronal network presents an obstacle to selectively measuring the activity of the same neuron over a long period of time. Here we report the use of nanopillar arrays to pin the position of neurons in a noninvasive manner. Vertical nanopillars protruding from the surface serve as geometrically better focal adhesion points for cell attachment than a flat surface. The cell body mobility is significantly reduced from 57.8 μm on a flat surface to 3.9 μm on nanopillars over a 5 day period. Yet, neurons growing on nanopillar arrays show a growth pattern that does not differ in any significant way from that seen on a flat substrate. Notably, while the cell bodies of neurons are efficiently anchored by the nanopillars, the axons and dendrites are free to grow and elongate into the surrounding area to develop a neuronal network, which opens up opportunities for long-term study of the same neurons in connected networks.

KEYWORDS Nanopillar, neuron, cell growth, cell migration, extracellular recording

A fundamental understanding of neural network formation, transmission, and remodeling requires measurements of individual neuron activities including firing threshold, firing rate, and temporal sequence.[1] Extracellular approaches such as patterned multielectrode arrays (MEA)[2−4] and planar field effect transistors[4] have been successful in simultaneously measuring multicell activities over an extended period of time and thus have provided valuable information regarding the development and formation of neuronal networks. For example, the emergence of synchronous electrical firing pattern and developmental changes of the network activity have been observed in networks of cultured cortical neurons.[5−7] In such studies, dissociated neurons obtained from fetal or neonatal brains are cultured atop the embedded electrodes or transistors. Electric signals generated by a neuron can be detected extracellularly if there is an electrode in close contact. However, it has been difficult to consistently measure the activity of the same neuron over a long-term period. This difficulty is partly due to neuron mobility and partly due to lack of neuron-to-electrode specificity.[8] Neurons cultured on a flat substrate tend to migrate over time, especially in the first few weeks.[9,10] The migration range can be as long as hundreds of micrometers and well beyond the detection range of a single electrode or transistor. As a result, patterned electrodes or transistors are not always monitoring the activity of the same neuron as neurons move around. This presents a challenge to monitoring individual neuron activities in a neuronal network for an extended time (up to months), which demands stable and specific neuron−electrode correspondence.

Considerable efforts have been put forth to control the migration of neurons and thus to improve the neuron−electrode interface.[11−16] The first approach is to promote neuron-to-electrode attachment by (1) chemical modification of the microelectrode surface via self-assembled monolayers of cell attractive or repulsive molecules[11,16] or (2) patterned deposition of adhesion-promoting proteins as by microcontact printing.[12−15] This approach makes micropatterns of adhesive molecules that promote neuron growth on the patterned electrodes. However, the current techniques do not perform consistently when the patterned features reach single cell scale. Each electrode rarely has just a single neuron growing atop; they generally have either no neurons or a cluster of neurons attached. In addition, axons and dendrites are also constrained to the patterned area, which limits free development of neural networks. The second approach involves fabricating neurocages/wells or picket fences that physically trap neuron cell bodies to stay in contact with the same electrodes.[8,17−19] Neuroncages developed in the Pine group were able to achieve one-to-one neuron-to-electrode correspondence and thus measure the activity of the same neuron over several weeks.[8] Picket fences developed in Fromhrez group were able to immobilize snail neurons on a silicon chip.[19] However, the fabrication of physical traps such as these requires complicated procedures and the process of loading one neuron per neurocage/trap can be tricky and time-consuming. The key features in those neurocages and fences are on the scale of multiple micrometers.

While previous techniques try to prevent neuron migration by chemically or physically confining them, we seek to engineer unique nanostructures that foster, rather than

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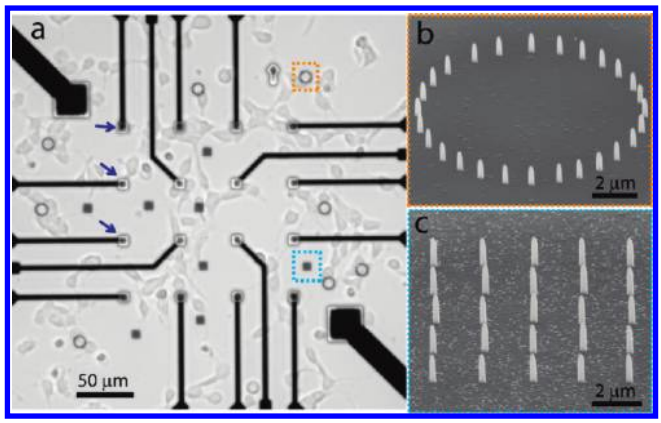

FIGURE 1. Illustration of cultured neurons on a nanopillar substrate. (a) A bright field image of neurons cultured on a MEA substrate with nanopillar arrays located both on the microelectrodes (blue arrows) and in open areas (orange and cyan squares). (b) SEM image of a ring-shaped nanopillar array. (c) SEM image of a 5 × 5 square nanopillar array.

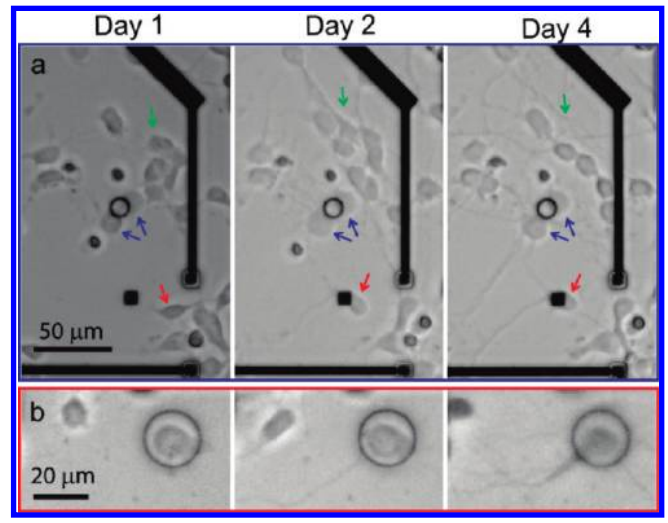

FIGURE 2. Migrations of cortical neurons are followed at 1 day, 2 days, and 4 days after plating. Over this 4 day period, neurons that are in contact with nanopillar arrays display significantly reduced mobility compared with those that are not. On the other hand, all neurons show similar morphology, survival rate, and neurite elongation rate. (a) Neurons that are not in contact with nanopillars (green arrows) are significantly more mobile over 4 days than those neurons in close contact with nanopillars (blue arrows). The red arrow points to a neuron that is very mobile in the first day on the flat surface before it comes into contact with a square nanopillar array and is arrested there. (b) A neuron initially plated inside a nanopillar ring is trapped inside.

impose, residence of neuron cell bodies atop the electrode of interest. In the past few years, a number of studies at the interface of nanotechnology and cell biology show that vertically aligned nanowires support cell attachment and survival.[20−27] Vertical nanowires were also shown to deliver large molecules such as proteins and DNAs into the attached cell.[21,22] Those studies indicate intimate and nondestructive interactions between vertical nanowires and biological cells. In this work, we explore the use of vertical nanopillars to reduce cell mobility on the substrate while maintaining normal function and activity of neurons. We find that vertical nanopillars serve as geometrically superior focal adhesion points for cell attachment and reduce the migration of neurons that are in contact with them. Individual neurons are pinned when by chance they are plated on the nanopillars or migrate to be in contact with them, and thus special cell-loading processes are not necessary. In addition, the small size of the nanopillar and the tendency of the cell membrane to wrap around the nanopillar indicate that each nanopillar, if serving as an electrode, would detect signals only from a single neuron. Therefore, nanopillars have the potential of recording the same neuron over an extended period of time.

Vertical nanopillars are fabricated by ion-beam or e-beam induced platinum deposition using a dual-beam focused ion beam (FIB)/scanning electron microscope (SEM) system. Platinum material is chosen for its biocompatibility and potential to directly measure the electrical activities of attached neurons. The dimensions of the nanopillars are typically 150 nm in diameter and 1 μm in height. The locations of the nanopillars can be precisely controlled. Nanopillar patterns are normally fabricated on top of the electrodes of a customized MEA substrate in order to anchor neurons at the electrode locations (Figure 1a). Nontransparent MEA electrodes make it difficult to observe nanopillars or neurons on top of them using optical microscopy. Thus, we also fabricate nanopillars in the transparent area of the fused quartz substrate to demonstrate the cell pinning effect of the nanopillars (enclosed by the blue and orange squares in Figure 1a). Two types of nanopillar patterns are tested—a ring-shaped circle with 10 μm diameter (Figure 1b) and a 5 × 5 square array with 2 μm spacing (Figure 1c).

Embryonic cortical neurons are isolated from E18 rats according to previously published protocols.[28] The nanopillar substrate is cleaned by oxygen plasma, sterilized in 70% ethanol, and coated with 1 mg/mL poly-L-lysine before cell plating. Dissociated neurons are plated on the nanopillar substrate and maintained in neurobasal medium supplemented with B27 and L-glutamine. Optical microscope images are taken every day to track the growth and migration of neurons on or off the nanopillars. Neurons that have cell bodies or neurites attached to the nanopillars display survival rate and cell morphology similar to those that are not in proximity to the nanopillars.

Figure 2 displays representative images that show distinct cell mobility for those neurons that are in close contact with the nanopillar arrays and those that are not. Neurons that are not attached to the nanopillars are mobile, and some migrate distances over a hundred micrometers in 4 days (green arrow in Figure 2a). In contrast, neurons in contact with the nanopillar patterns exhibit significantly hindered movement. For example, two neuron cell bodies attached to a nanopillar ring (as indicated by the blue arrow in Figure 2a) are essentially stationary over the 4 day period, despite

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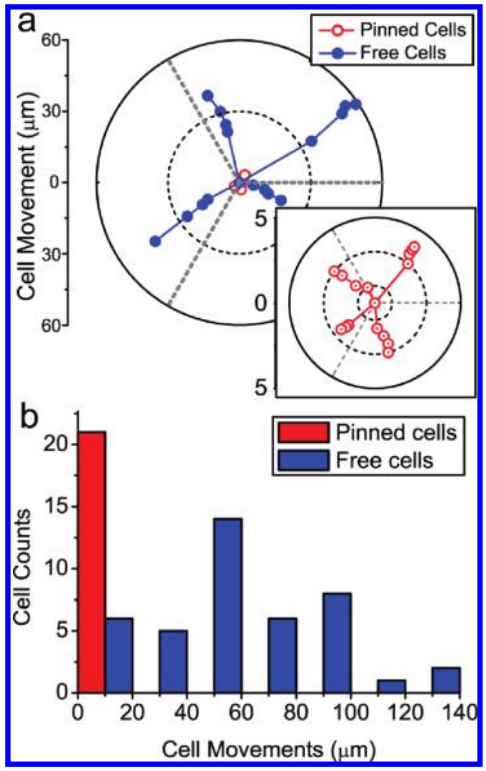

FIGURE 3. Statistics of cell migration over 5 days. (a) Typical movement traces of four nanopillar-pinned and four free-migrating neurons. Bottom right plot shows zoom-in of the nanopillar-pinned cell movements. While the free-migrating neurons explore ~60 μm distances, the nanopillar-pinned cells move no more than 5 μm. (b) Analysis of neuron movements over 42 free-moving and 21 nanopillar-pinned neurons show that nanopillars effectively stopped the migration of neurons.

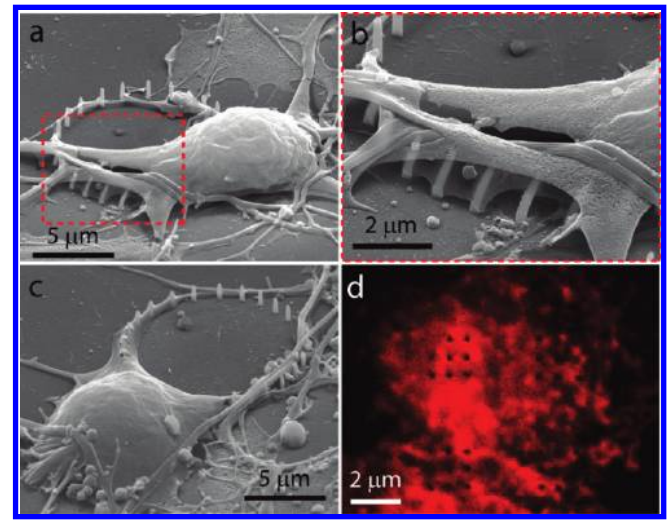

FIGURE 4. Examining the neuron−nanopillar interface by SEM and fluorescence microscopy. (a). An SEM image of a neuron growing atop a ring of nanopillars shows close contact between neurons and nanopillars. (b). Zoomed-in picture of (a) shows that cell membrane tightly wrapped around nanopillars. The interaction between the nanopillars and the neurons seem to exert forces on the nanopillars so as to bend some of them. (c). An SEM image of a neuron with one of its neurites preferentially growing along the ring-shaped nanopillar arrays. (d). Confocal microscope image of immunostained actin filaments shows that nanopillars (black dots) are imbedded in the cytoskeletal network.

extending out long axons. The red arrow points to a neuron that migrates freely on the first day before reaching a nanopillar square array on the second day. Over the next 2 days, the neuron significantly elongates its neurites but its cell body stays at the same location. In Figure 2b, a neuron plated inside a nanopillar ring at the very beginning stays inside the ring for the whole period.

Typical migration traces of four neurons on the flat substrate and four neurons attached to the nanopillar arrays are plotted in Figure 3a in a polar graph. From the center outward, these data points represent center locations of cell bodies from day 1 to day 5, relative to the location of their cell bodies on day 1. It is obvious that the migration of neurons is effectively inhibited by nanopillars. While neurons on flat substrate migrate on-average 60 μm in 5 days, nanopillar-pinned neurons are mostly confined within a 5 μm range. Even this movement occurs mostly on the first day after pinning, during which the neurons move to increase their attachment to the nanopillars. Afterward, the pinned neurons move very little. In Figure 3b, statistics of neuron migration distances are summarized. The cell movement is calculated as the sum of each neuron's movement every day. After 5 days of observation, free-migrating neurons counted over 42 cells show a mean movement of 57.8 μm, while nanopillar-pinned neurons counted over 21 cells only have a mean movement of 3.9 μm.

In order to closely examine the nanopillar−neuron interface, neurons cultured on the nanopillar substrate are fixed and imaged by SEM (Figure 4a−c). Five days after plating, cells are first fixed by glutaraldehyde treatment and then stained with osmium tetroxide staining for contrast enhancement. The sample is dehydrated via CO_2 critical point drying in order to preserve cell morphology. The shape of the neurons suggests that they not only survive on top of the nanopillars but also preferentially grow attached to the nanopillars (Figure 4a−c). Figure 4c illustrates the morphology of a neuron growing just outside a ring of nanopillars. The neuron and its projections show a relative preference to attach and grow on nanopillars versus the flat substrates. The fact that cells usually shift to increase their attachment to the nanopillars once they reach them also implies the same preference. Similar behavior has been observed in some previous research.[24,27]

Nanopillars are usually tightly engulfed by neurons. Even neuritic protrudings try to increase their contact with nanopillars by wrapping them with a thin sheet of membrane (Figure 3b). Some of the nanopillars embedded in the cell are bent, possibly due to a force generated by the cell attachment. The bending is not caused by the SEM sample preparation process because it is likewise noted by optical microscopy with live cells. It is conceivable that the engulfment of the nanopillars is aided by cytoskeletal elements such as actin filaments and microtubules, which

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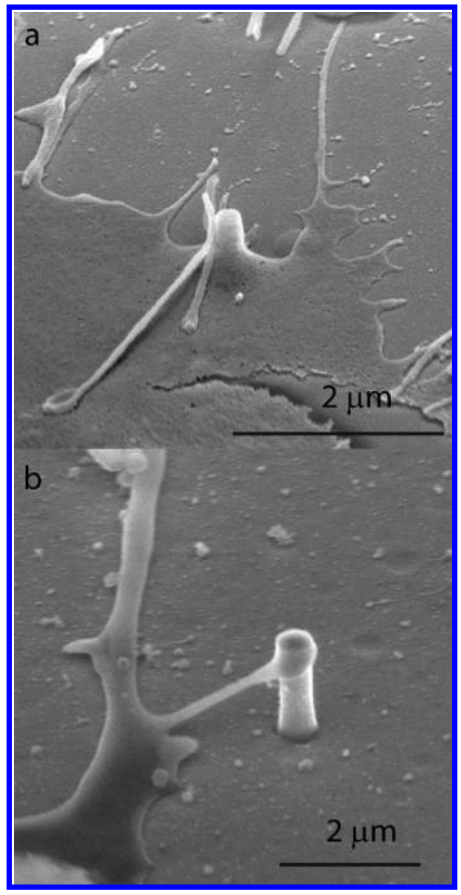

FIGURE 5. SEM of cells cultured on Si and SiO_2 nanopillar substrates. (a) An SEM image shows a SiO_2 pillar engulfed by the cell membrane. (b) An SEM image shows a protruding of a neuron reaching a Si nanopillar.

generate mechanical tension around the nanopillars and bend them. To further evaluate the interaction between the nanopillars and the cytoskeleton, neurons are fixed and actin filaments are labeled red with TRITC-conjugated phalloidin. Nanopillar arrays show as black dots in the confocal fluorescence image (Figure 4d). The focal plane of the confocal fluorescence image was set at the middle of the nanopillars with *z*-slice about 0.5 μm. The confocal image confirms that the nanopillars are embedded into the actin cytoskeleton network.

Next, we investigate whether the cell pinning effect is due to the geometry of nanopillars or due to the surface properties of the platinum material. To test this, we fabricate nanopillars with similar geometry but using Si or SiO_2 instead of Pt. Si and SiO_2 nanopillar structures are fabricated by reactive ion etching using Au or SiO_2 nanoparticles as masks. The fabrication process and the subsequent removal of particle masks are carried out as previously described.[22] The attachment and growth of cortical neurons on the Si and SiO_2 nanopillar substrates are similar to those on the Pt nanopillar substrate. SEM analysis (Figure 5) of neurons growing on Si and SiO_2 nanopillar substrates also shows strong interactions between the cell and the nanopillar. Notably, neurite protrudings, which are usually involved in guiding axon elongation and neuron migration, often show a strong tendency to fix their ends on nanopillars, as illustrated in Figure 5b. This behavior may explain why neurons preferentially migrate toward nanopillars. The fact that similar behavior is observed for nanopillars of all three materials indicates that the pinning effect of nanopillars is likely a geometry effect, rather than a material effect. A conceivable mechanism is that nanopillars protruding from the surface can serve as focal adhesion points. They constitute stronger anchor points for the cell matrix than those formed on a flat surface.

In summary, we report the use of nanopillar arrays to inhibit the migration of attached neurons. Neurons in close contact with the nanopillars show significantly reduced mobility and are essentially pinned to the nanopillars. Despite this pinning effect, neurons growing on nanopillars show similar growth patterns to those seen on a flat substrate. Within the parameter regime that we have tested (75−400 nm in diameter and 700 nm to 2 μm in height), cell survival rate and the pinning effect do not seem to depend on the size of the pillars. If patterned on top of microelectrodes, vertical nanopillars would serve as neuron traps for long-term neuronal network study with MEA and also improve the neuron-to-electrode contact.

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