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Published in:
ACS Applied Materials and Interfaces

DOI:
10.1021/acsami.5b03798

2015

ACS APPLIED MATERIALS & INTERFACES

Research Article

www.acsami.org

Support of Neuronal Growth Over Glial Growth and Guidance of Optic Nerve Axons by Vertical Nanowire Arrays

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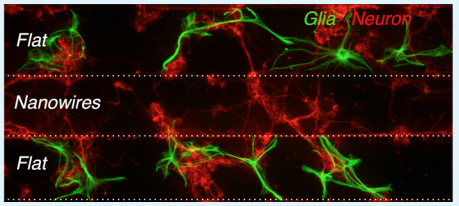
Supporting Information

ABSTRACT: Neural cultures are very useful in neuroscience, providing simpler and better controlled systems than the *in vivo* situation. Neural tissue contains two main cell types, neurons and glia, and interactions between these are essential for appropriate neuronal development. In neural cultures, glial cells tend to overgrow neurons, limiting the access to neuronal interrogation. There is therefore a pressing need for improved systems that enable a good separation when coculturing neurons and glial cells simultaneously, allowing one to address the neurons unequivocally. Here, we used substrates consisting of dense arrays of vertical nanowires intercalated by flat regions to separate retinal neurons and glial cells in distinct, but neighboring, compartments. We also generated a nanowire patterning capable of guiding optic nerve axons. The results will facilitate the design of surfaces aimed at studying and controlling neuronal networks.



KEYWORDS: *nanowires, gallium phosphide, retina, CNS, glia, neurons*

INTRODUCTION

Primary neural cell cultures are widely used to investigate features of the central and peripheral nervous systems (CNS, PNS) since they offer a highly controllable environment compared to an *in vivo* neural system. Such cultures can be used to examine the influence of specific molecules on, e.g., cell survival, differentiation, and interactions, for drug development or the study of potentially neurotoxic compounds.[1,2] The effects of the manipulations can be assessed, for instance, by immunocytochemistry or techniques that allow visualization of live cells. Functional characteristics can also be evaluated through calcium imaging or multielectrode arrays (MEAs) that permit the simultaneous monitoring of several neurons, opening up new neural culture applications, such as biosensing.[1,2]

Neuronal survival and function are greatly dependent on glial cell support,[3] and it has been observed even *in vitro* that glial cells control the number of synapses and increase the synaptic efficacy in neuronal cultures.[4,5] Direct proximity of neurons to glial cells has also been shown to be necessary to support long-term potentiation, a mechanism that (*in vivo*) underlies learning and memory.[6] Thus, to ensure that cultured neurons behave as closely as possible to neurons in the *in vivo* situation, it is important that they are cultured together with glial cells.

In cultures of neural tissue, cells are often dissociated, which activates the glial cells, leading to a glial overgrowth[7] that can obscure the neurons, making it difficult to access them for electrophysiological analysis. This issue can be circumvented by adding antimitotic drugs to control glial cell proliferation; however, this has been shown to also affect neuronal survival, thus resulting in an inferior model representativeness.[8] Moreover, in the case of surface-integrated electrodes such as MEA, where it is crucial to achieve close contact between electrodes and neurons, the adhesion of glial cells on the substrate can limit the use of the electrodes for neuronal signal detection.[9−12]

One approach to address this problem consists of confining astrocytes to a distinct area in close vicinity to the neurons, e.g., by using microfluidic devices with distinct culture chambers for glial cells and neurons[13] or by using surface chemistry patterning.[14,15] However, the use of microfluidic compartments limits the access to the cells, while chemical patterns, although successful at confining neurons and astrocytes in different places on the substrate, rely on polymer and protein adsorption on the surface, which may not be stable over time. Moreover, in the latter, neurons and astrocytes have to be cultured in subsequent steps making it cumbersome to implement. There is therefore a need to develop a stable surface patterning, that enables a good separation when coculturing neurons and glial cells simultaneously.

We have cultured CNS cells on gallium phosphide (GaP) substrates and reported recently that, while both neurons and glial cells survive on the material, neurite outgrowth is exceptionally improved when culturing the cells on arrays of

Received: October 15, 2014
Accepted: August 11, 2015
Published: August 11, 2015

DOI: 10.1021/acsami.5b03798
ACS Appl. Mater. Interfaces 2015, 7, 18944−18948

randomly positioned vertical GaP nanowires.[16] In the latter, neurons were also found to express synaptophysin, indicating that the cells contained synaptic vesicles and are capable of making contacts with surrounding neurons and form networks.[16]

In the present study, we used patterned GaP nanowire arrays and a single cell-culture step to obtain a separation of retinal neurons and glia in distinct neighboring regions on the substrate. Furthermore, we have tested whether it is possible to guide the growth of neurites using rows of vertical GaP nanowires, as previously shown for PNS processes.[17,18] Such features would enable, for instance, the control of the neuronal-network architecture and the interrogation of specific axons when using MEA.

RESULTS AND DISCUSSION

We have employed GaP substrates with patterns of vertical GaP nanowires, realized using electron beam lithography and metal organic vapor phase epitaxy, as described previously[19,20] (see Supporting Information for detailed experimental protocol). In order to obtain a separation of neurons and glial cells, we designed regions with different topographies on GaP substrates (Figure S1). In one set of experiments, mouse retinal cells were cultured for 8 and 18 days *in vitro* (DIV) on substrates consisting of 100 μm wide bands containing dense vertical arrays of nanowires separated by 100 μm wide regions with a flat topography (100/100-dense). Using our protocol, both single cells and cell clusters could be seen on both nanowire and flat regions. This cell distribution is the same as observed in one of our previous studies,[16] in which the GaP substrates were not patterned (i.e., they were either flat or contained randomly positioned nanowires), and is the same distribution we obtain when the cells are cultured on standard polystyrene culture substrates (Figure S3). This indicates that GaP nanowires (randomly positioned or in bands separated by flat regions) do not have a negative effect on cell distribution. It should be noted, however, that this is not a general property of nanowires, as we have shown in another study that, when the same protocol was used to grow the cells on silicon nanowires, the cell distribution and other parameters were compromised by the presence of contaminants entrapped in the material.[21]

In the present study, neuronal cell bodies stained with β-tubulin III were seen to attach on both flat and nanowire regions (see the Supporting Information for detailed cell culture and labeling protocols). It was not possible to quantify the exact number of β-tubulin III cells on each surface since many of these cells appeared in the clusters. It was similarly difficult to estimate the exact number and direction of labeled neurites, but the analysis we have performed showed that these extended mostly in association with the nanowires, crisscrossing the arrays (Figures 1 and 2a), which conforms to our previous observation that GaP nanowires have a positive effect in promoting neurite outgrowth in retinal cell cultures.[16]

In addition, we observed in the present study an agglomeration of processes aligned on top of the outermost row of each nanowire array (Figures 1 and S4). The good support of neurite outgrowth on GaP nanowires has been linked to the adsorption of extracellular matrix and neurotrophic molecules on the nanowires (derived from the culture medium and the cells themselves).[16,22] It is thus possible that the growing neurites stay away from the flat surfaces due to the lack of roughness and/or lower expression of growth-promoting molecules, aligning instead along the edges of the arrays.

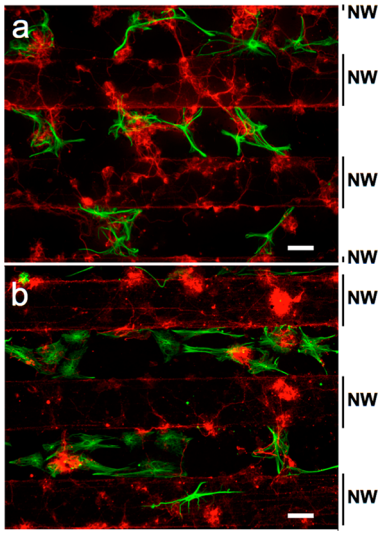


Figure 1. Retinal cells cultured on 100/100-dense substrates. Neuronal cells (β-tubulin III, red) and glial cells (GFAP, green) on 100 μm wide bands with dense arrays of nanowires (indicated by vertical lines and "NW") separated by 100 μm wide bands with a flat topography after 8 DIV (a) and 18 DIV (b). Scale bar, 50 μm.

In contrast to what was observed with the neuronal cells, labeling with GFAP revealed that glial structures were predominantly found within the 100 μm wide flat regions and were only rarely observed over the nanowires (Figures 1 and 2b). In most cases, it was not possible to unequivocally determine the actual number of cells associated with GFAP labeling (see Figure S5 for an illustration of a GFAP positive structure). For this reason, we have counted the number of GFAP positive structures, which could correspond to one or more, intermingled, cells. Even considering that this approach may have resulted in an underestimation of the number of glial cells, the analysis revealed that 89% ± 4% (mean ± standard error of the mean) and 84% ± 3% (mean ± standard error of the mean) of all identifiable GFAP positive structures are located on the flat regions at 8 and 18 DIV, respectively. The mechanisms underlying this accumulation of glial cells and processes are not entirely clear. It is assumed that glial cells were evenly distributed over the entire substrate immediately following seeding. The possibility that glial cells migrated away from the nanowire regions may be excluded since previous studies have shown that cells are in fact less motile on nanowires than on flat surfaces.[23,24] A difference in glial cell adhesion between nanowire and flat regions appears also not to be the predominant factor since a few glial cells could be seen attaching on nanowire regions. Glial cells, in contrast to neurons, retain the ability to divide throughout life in response to a number of different stimuli.[25] We have recently shown that fibroblast proliferation was significantly reduced on GaP nanowires compared to control flat substrates.[24] In the present study, the glial structures found on the flat regions were often seen in groups of adjacent cells (Figures 1 and 3), suggesting that at least some of them may have derived from proliferation postseeding. It is thus possible that the higher incidence of glial cells in the flat regions stems from a higher proliferation rate in these regions. This would appear to be contradicted by the observation that similar proportions of GFAP labeled structures

DOI: 10.1021/acsami.5b03798
ACS Appl. Mater. Interfaces 2015, 7, 18944−18948

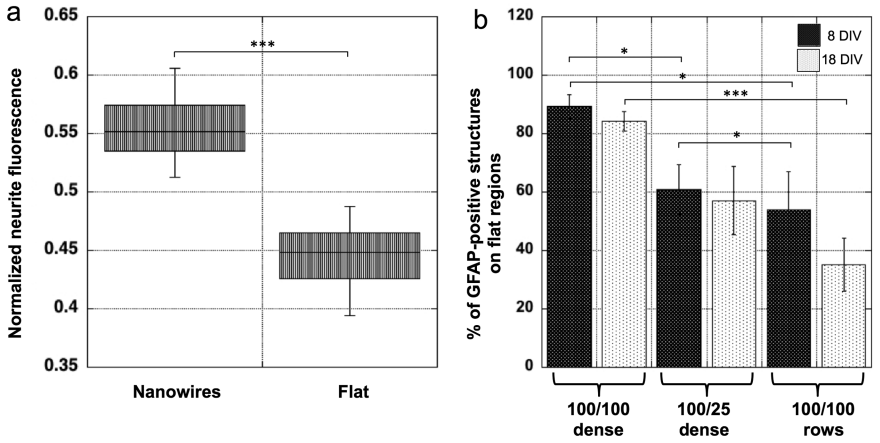


Figure 2. (a) Quantification of neurite outgrowth on the 100/100-dense substrate. Normalized β-tubulin III fluorescence intensity (clusters excluded) on nanowire areas and flat areas, averaged over all samples. Mean value ± standard error of the mean ($_{***}p < 0.001$, Mann-Whitney). (b) Percentage of GFAP positive structures on the flat regions for 100/100-dense substrates, 100/25-dense substrates, and 100/100-rows substrates. Mean value ± standard error of the mean ($_{*}p < 0.05$, $_{***}p < 0.001$, Mann-Whitney).

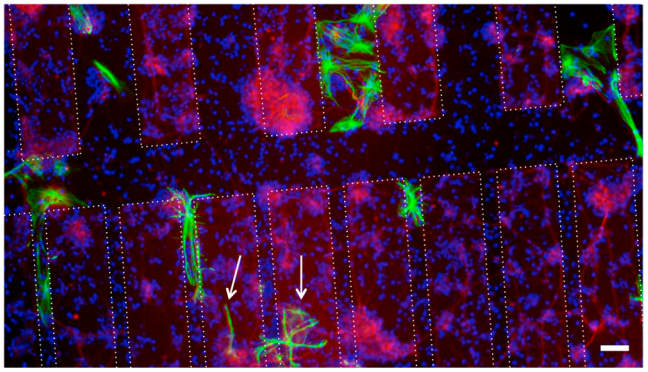


Figure 3. Retinal cells cultured on substrates with mixed patterns. Neuronal cells (β-tubulin III, red) and glial cells (GFAP, green) cultured for 18 DIV on substrates consisting of 100 μm wide nanowire regions (dashed line) separated by 100 μm wide flat regions (100/100-dense, top of the image) and 25 μm wide flat regions (100/25-dense, bottom of the image). Cell nuclei are labeled with DAPI (blue). Scale bar, 50 μm.

were observed on flat regions at both 8 and 18 DIV. However, we noted also a change in the morphology of the glial structures over time (see Figure 1), which made the identification of individual cell profiles at 18 DIV yet more difficult. In any case, our results indicate that it may be possible to design substrates where neurons and glial cells grow in separate, but neighboring, compartments defined by distinct surface topographies.

In order to maximize the surface area dedicated to interfacing neurons on the substrate, we tested whether the size of the flat region could be reduced, without loosing the neuron-glia separation. For this purpose, we designed a substrate containing two sets of patterns, one with 100 μm wide nanowire regions separated by 100 μm wide flat regions (100/100-dense) and another with 100 μm wide nanowire regions separated by 25 μm wide flat regions (100/25-dense), and cultured retinal cells on the substrates for 18 DIV (Figure 3). We observed more GFAP positive structure glial cells adhering on the nanowire regions when these were only 25 μm apart (Figure 3, white arrows). For this spacing, only 61% ± 9% (mean ± standard error of the mean) and 57% ± 12% (mean ± standard error of the mean) of the glial structures were found on the flat regions, at 8 and 18 DIV, respectively (Figure 2b). Glial cell processes also extended onto the nanowire regions more often than they did when the nanowire arrays were 100 μm apart (Figure 3). Reducing the distance between the nanowire arrays, while providing a larger area for interfacing neurons, expectedly also increases the probability of glial cells attaching to the nanowires. The observations therefore suggest that, of the two configurations, the 100/100-dense would be the most suitable for the purpose of culturing neurons and glial cells in close vicinity but in distinct regions of the substrate. Such a configuration would be useful, for instance, in systems aiming at the interrogation of neuronal circuits using MEAs.

Neuronal contact guidance has been extensively studied over the past years (reviewed in refs 26 and 27), and a few years ago, we demonstrated that rows of nanowires can guide axons from the peripheral nervous system.[17,18] In the present study, we examined whether also the outgrowth of CNS neurites could be guided using the nanowires. For this purpose, we designed a pattern consisting of repetitions of 100 μm wide nanowire regions (spaced by 100 μm wide flat regions), in which the nanowires were arranged in rows spaced 10 μm apart (100/100-rows, Figure S1b), rather than in a dense regular array. At 18 DIV, neuronal processes labeled using the antibody against β-tubulin III appeared guided by the nanowires (Figures 4 and S6). The degree of polarization of the neurites can be appreciated when comparing with neurite outgrowth on flat and randomly positioned nanowire substrates (Figure S7).

Using an antibody against TRPV4, a functional cation channel, we observed that at least some of the processes aligned along the rows in 100/100-rows substrates were stained and are therefore likely to correspond to processes of retinal ganglion cells (Figure 5), which we have shown express this protein.[16] These observations suggest that it may be possible to promote regeneration and direct the outgrowth of retinal ganglion cell axons by using rows of vertical GaP nanowires.

In contrast to what was observed with dense regular nanowire regions, no clear neuron−glial separation was obtained when the nanowire region consisted of rows, despite the presence of 100 μm wide flat surfaces (Figure 4). In these substrates, glial cells could also be seen adhering to the nanowire regions, with some processes similarly oriented along the nanowire rows; 60% ± 9% (mean ± standard error of the mean) of glial structures adhered on the flat area at 8 DIV and

DOI: 10.1021/acsami.5b03798
ACS Appl. Mater. Interfaces 2015, 7, 18944−18948

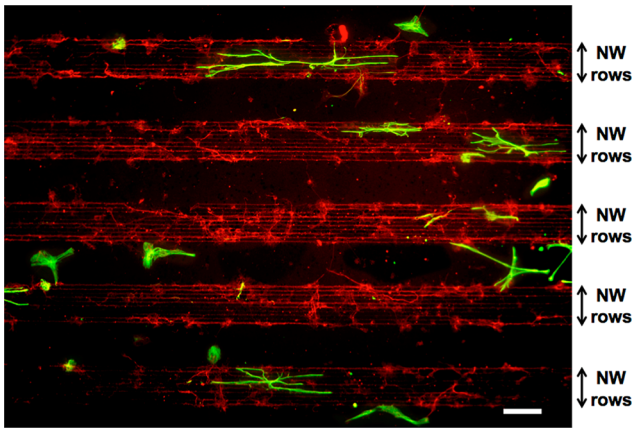


Figure 4. Retinal cells cultured on 100/100-rows substrates for 18 DIV. Neuronal cells (β-tubulin III, red) and glial structures (GFAP, green) on regions with rows of nanowires (NW rows) and flat regions. Scale bar, 100 μm.

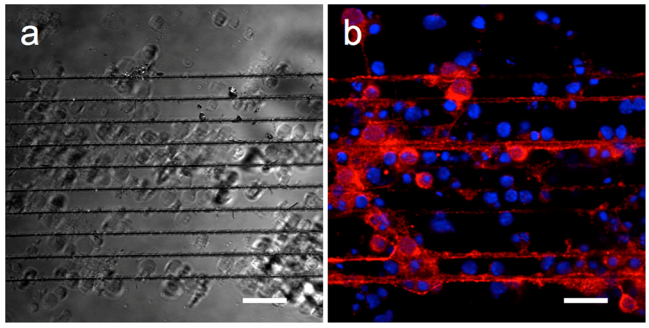


Figure 5. Retinal ganglion cell axons guided on 100/100-rows substrates. Confocal microscopy image of the nanowires acquired using the laser reflection mode (a) and confocal fluorescence microscopy image of retinal ganglion cells (TRPV4, red) and cell nuclei (DAPI, blue) after 18 DIV (b). Scale bars, 20 μm.

35% ± 10% (mean ± standard error of the mean) at 18 DIV (Figure 2b). It is possible that the conditions afforded by the regular array, which seemed to limit glial presence on nanowires, are not present if the distance between the nanowires is increased beyond a certain point.

CONCLUSION

The present work shows that it is possible to fabricate substrates for interfacing CNS neurons using vertical nanowires, such that neurons and glial cells are kept in close, but separate, compartments. The study shows in addition that, depending on the arrangement of the nanowires, it is also possible to guide the elongation of CNS neuronal processes. Conceptually, in the context of using vertical nanowire arrays in, for instance, MEA systems, nanowire rows may be used to control the network architecture by guiding neurites between two electrodes, while dense arrays of nanowires may be used in the direct vicinity of the electrodes to limit or prevent an accumulation of glial cells. Further, glial cell activation and proliferation are also induced following the implantation of neuroprosthetics, significantly reducing their performance.[28] In future studies, it would be interesting to investigate whether patterned vertical nanowire arrays can be used in CNS implants to reduce the extent of glial cell encapsulation, improving the biocompatibility and functionality of these implants.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b03798.

Detailed experimental protocols and SEM and fluorescence microscopy images (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors would like to thank Shantonu Biswas for help with improving the EBL process, as well as Henrik Persson and Karl Adolfsson for nanowire growth. Gaëlle Piret was financed by a postdoctoral grant from the Lund Tekniska Högskola (LTH) at Lund University. The study was supported by NanoLund, the Swedish Research Council, VELUX STIFTUNG, Crown Princess Margareta's Committee for the Blind, Stf för Synskadade i fd Malmöhus Län, Edwin Jordans Stiftelse för Oftalmologisk Forskning, Gun och Bertil Stohnes Stiftelse, Greta och Johan Kocks Stiftelse, Magnus Bergvalls Stiftelse, and the Neuronano Research Center at Lund University.

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