Review

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Neurons on Nanotopographies: Behavioral Responses and Biological Implications

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Neural cells are well known to be affected by the topographical features of the surfaces to which they adhere. There have been numerous reports showing guided neurite extension on microgrooves and micropillar arrays made of a wide range of materials. However, it has recently been disclosed that neural cells on nanotopographies exhibit much more derivatized and complicated responses than just neurite guidance. Nevertheless, the biological mechanisms for these responses are not yet understood. In this review, we categorized the responses of neurons to nanotopographies into three groups (adhesion, neurite guidance, and developmental acceleration). In addition, we also tried to elicit biological implications about the mechanisms for the recognition of nanotopographies. Further investigation of neuronal responses to nanotopographies would highly inspire both fundamental research about neuronal development and practical applications related to neuro-regeneration.

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1. INTRODUCTION

Neurons, like other cell types, receive complicated stimuli from their surrounding environments. These external perturbations play crucial roles in the survival, development, and functionality of neurons, as well-exemplified by soluble growth factors or proteins immobilized on the surfaces of extracellular scaffolds or other cells. Biospecific recognitions of these biochemical stimuli are fulfilled by corresponding receptors on neuronal membranes, resulting in the onset of numerous essential intracellular signaling pathways. The intracellular machinery for the biospecific recognitions has been a primary target in molecular and cellular neurobiology, and has proven to be involved in most neuronal functions and behaviors, including synaptogenesis/synaptic plasticity,[1,2] migration,[3] neurite pathfinding,[4,5] and neural development and differentiation.[6]

However, the existence of the intracellular machinery that actively recognizes the physical attributes of surrounding environments is yet uncertain, although neuronal behaviors are obviously influenced by surface topography, stiffness, or physical tension. The most representative example occurs in context of the neuronal migration within the developing nervous system, where neurons migrate in a direction guided by the morphology of pioneering glial cells or fiber structures in the extracellular matrix (ECM).[3,7] The ECM is mainly composed of collagen- or elastin-based fibers decorated with smaller proteins and peptidoglycans, which in turn formulate micro/nano hierarchical topography.

Attempts to elucidate topographical effects on neuronal behaviors originated from the observation of contact guidance: cells were shown to migrate or develop in a direction dictated by the physical morphology of the surfaces on which they attached. The majority of reports about contact guidance of neural cells started to

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emerge several decades ago, as microfabrication techniques allowed the systematic generation of microtopographies with controllable features. One example was a substrate with lithographically fabricated microgrooves, on which hippocampal neurites were aligned either parallel or perpendicular to the grooves according to the type of neurons and the depth of the grooves.[8,9] Since this simple observation, there have been increasing reports regarding neuronal behaviors on a variety of microtopographical features, represented by pillars,[10,11] grooves,[12–15] and even a molded glial cell surface.[16] On these microtopographies, guided neurite extension on/between the features were commonly observed.

More recently, it became possible to investigate neuronal behaviors on nanotopographical substrates, by virtue of the emergence and development of nanofabrication technologies. On nanotopographies, more derivatized responses including advanced adhesion and enhanced neurite development were additionally observed, as well as guided neurite extension. This was worth considering, because it was highly likely that nanotopographical stimuli, apart from microtopographies, would affect neurons

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in a sub-cellular fashion and induce bottom-up changes in cellular-scale behaviors. It also implied that neurons might actively recognize nanotopographies by using their innate machinery. However, the biological mechanism for this specific recognition of nanotopographies is not yet established.

In this article, we sought to review recent publications regarding neuronal responses to nanotopographical surfaces. We have categorized neuronal behaviors on various nanostructures and also have considered the influences of the types of neurons or nanotopographies on them. We also provided a few reports that elucidated biological mechanisms of neuronal responses to nanotopographies and future perspectives related to them.

2. RESPONSES OF NEURAL CELLS TO NANOTOPOGRAPHIES

Advanced micro/nanofabrication technologies have enabled the generation of numerous topographies recapitulating the morphological aspects of native tissue environments, and on these numerous responses made by a variety of cell types were reported, as well-summarized by other reviews.[17,18] However, the responses of neural cells were distinguished from those of others, because they have distinct behavioral characteristics: neural cells generally do not proliferate or differentiate, and they develop in a polarized shape to make unidirectional connections with each other. Particularly, they continuously expand in a form of neurite elongation until they get sufficiently maturated, and thus the elongating tips of neurites implement pathfinding tasks. The scale of topography also deserves consideration, as it decides whether the topographical stimuli that neurons would receive are on the cellular or subcellular scale. In this review, we focused on three responses of various types of neural cells to the substrates with nanoscale (10 nm–1 μm) structures (Fig. 1, Table I).

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2.1. Adhesion, Attachment, and Viability

Adhesion and survival are relevant to all types of adherent cells, and the changes on them are probably the most studied effect of nanotopographies on cells. This was also the case for neural cells; neurons adhered and survived on nanotopographical substrates without adhesive coatings, whereas the coatings were strongly required for the adhesion of neurons on flat surfaces. When the adhesive coatings were added on the nanotopographies, there were synergistic advances in both adhesion and viability in comparison to flat substrates with coatings.

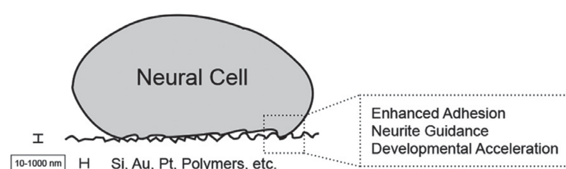

Figure 1. Neurons receive subcellular-scale stimuli from nanotopographies and exhibit intracellular changes.

The results were mainly shown in the studies using substrates with etched nanoroughness. Bayliss et al. investigated the adherence and viability of rat neuronal (B50) cells seeded on nanostructured porous Si, where B50 cells showed higher adherence and viability than on the polycrystalline and bulk Si.[19] Rough Si substrates were also used for culturing substantia nigra neurons, which adhered to the surface with an average roughness (R_a) ranging from 20 to 70 nm.[20] In this range, neurons adhered to the surface and survived for over 5 days without neuro-adhesive coatings. In addition, neurons even migrated to regions of optimum roughness if the substrates were micropatterned with different roughnesses.[21] Similar results were obtained from cortical neurons seeded on the Si surface with three different R_a, 18, 64, and 204 nm, where the best neural adhesion occurred on the Si wafers with surface R_a in the neighborhood of 64 nm.[22] The results from Si nanoroughness indicated that neuronal attachment was considerably moderated by changing surface roughness, which was supported by the observation of the interactions between the neural cells and Si substrates using atomic force microscopy (AFM), in which the increase of friction forces on the boundary around the neurons was measured.[23]

These roughness-responding behaviors were not restricted to Si substrates, but the nanoroughness made by titanium nitride (TiN) films also assisted the adhesion of primary hippocampal neurons.[24,25] The adhesion of neural cells were additionally studied on nanoporous substrates, but it turned out that the effects of nanopores to neuronal adhesion and viability were not differentiable from those of nanoroughness, as shown by B50 cells on stain-etched porous Si,[26] PC12 cells on gold nanopores[27] or porous Pt structures,[28] and human neuroblastoma (SK-N-SH) on anodized porous Si.[29] In the case of human neuroblastoma (SH-SY5Y) on gold substrates, however, the cells preferentially adhered on flat surfaces other than nanorough gold surfaces (Fig. 2(a)).[30] This unique nanotopography-rejection of neuroblastoma cell lines needs to be investigated by further studies.

Inspired by nanoroughness studies, there was an issue of culturing neural cells on vertically grown nanowires, since Hällström et al. reported that dorsal root ganglia (DRG) neurons adhered and survived normally on vertically grown GaP nanowires without neuro-adhesive coatings, although complete penetration of the cells by nanowires was observed (Fig. 2(b)).[31] To address this, the biocompatibility of other materials-based nanowires including gold, Si, Ge, SiGe, and GaN for hippocampal neurons was confirmed,[32,33] and using this neuro-adhesiveness of nanowires, there was an effort to physically pin cortical neurons by using SiO_2 nanopillar arrays in order to locate

Table I. Summary of neuronal responses to nanotopographies.

Response	Topography	Materials	Neuron types	References
Adhesion	Nanoroughness	Si	Substantia nigra	[20, 21]
			Cortical	[22]
			Hippocampal, cortical neurons	[23]
		TiN	Hippocampal neurons	[24, 25]
		Au	Neuroblastoma (SH-SY5Y)	[30*]
	Nanopore	Si	Rat B50	[19]
			Rat B50	[26]
			Neuroblastoma (SK-N-SH)	[29]
		Au	PC12	[27]
		Pt	PC12	[28*]
	Nanowire	GaP	DRG	[31]
		Si, SiGe, Ge, GaN, ZnO, Au	Hippocampal neurons	[32, 33]
		Pt, Si	Cortical neurons	[34, 35]
Neurite Guidance	Nanogroove	Polymer (PMMA)	DRG	[37*]
		Polymer (Polyurethane acrylate)	Neuroblastoma (N1E-155)	[38*]
		Polystyrene	PC12	[39, 45]
		Polymer (COC)	PC12	[40*, 41*, 42*, 43, 44]
			F11	[46]
		Polymer (Azopolymer)	PC12	[47]
		Polymer (PLLA)	DRG	[48, 51]
			Primary motor neurons	[49, 50]
			PC12	[52*]
	Nanofiber	Polymer (PCL)	DRG	[53*, 55*]
		Polymer (PLLA & PCL)	DRG, PC12	[54*]
		Polymer (PMMA)	DRG	[56*]
Developmental Acceleration	Nanotube	CNT	Hippocampal neurons	[57, 58, 59, 60]
			PC12	[61]
	Nanofiber	Polymer (Polyamide)	Various types	[62]
		Silk fibroin	Various types	[63]
	Nanogroove	Si	PC12	[64]
	Nanopore	Si	DRG	[65, 66]
		AAO	Hippocampal neurons	[67]
	Nanobead	Si	Hippocampal neurons	[69]

Note: *Longer neurite outgrowth was also shown at the same time.

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them properly on microdevices (Fig. 2(c)).[34] On these nanopillars, the interface interactions between cortical neurons and the nanopillars were investigated by transmission electron microscopy (TEM), and they observed that the cells wrapped around the pillars and grew on top of them.[35] In addition, there was an interesting report by Park et al. who used the cell-penetrating ability of nanowires to deliver exogeneous biomolecules into the cells.[36] Taken together, surface nanotopographies (nanoroughness, nanopores, and nanowires) obviously supported the adhesion and survival of neural cells, regardless of the type of neurons and the materials constructing the topographies. In particular, it was shown that neuro-adhesive coatings are not required for the adhesion of neurons on those nanotopographies.

2.2. Directional Guidance of Neurite Outgrowth

The guidance of neurite outgrowth is the most significant and well understood phenomenon between neural cells and topographies because it is a unique response of neural cells and also highly impactful for designing *in vitro* neuronal networks or neural regeneration applications. Over the past several decades, it has been extensively reported on various types of elongated microstructures such as microgrooves or micropillar arrays, and neurites on these microstructures were observed to grow between/along the structures or avoid them. Recently, however, it is becoming clear that the guidance effects also can be realized on elongated nanostructures despite the huge discrepancy from microstructures in scale. Moreover, in most cases on nanotopographies the guidance was accompanied by improved adhesion or neurite extension.

For example, nano-imprinted patterns of polymethylmethacrylate (PMMA)-covered silicon surfaces were used for a DRG neuron culture. The patterns consisted of parallel grooves with a depth of 300 nm, widths of 100–400 nm, and spacings of 100–1600 nm. On these patterns, most axons displayed contact guidance on all patterns down to 100 nm and preferred to grow on the ridge edges.[37] Similar results were obtained by culturing N1E-155 neuroblastoma cells on laminin-coated polyurethane acrylate grooves, showing highly aligned neurite outgrowth along

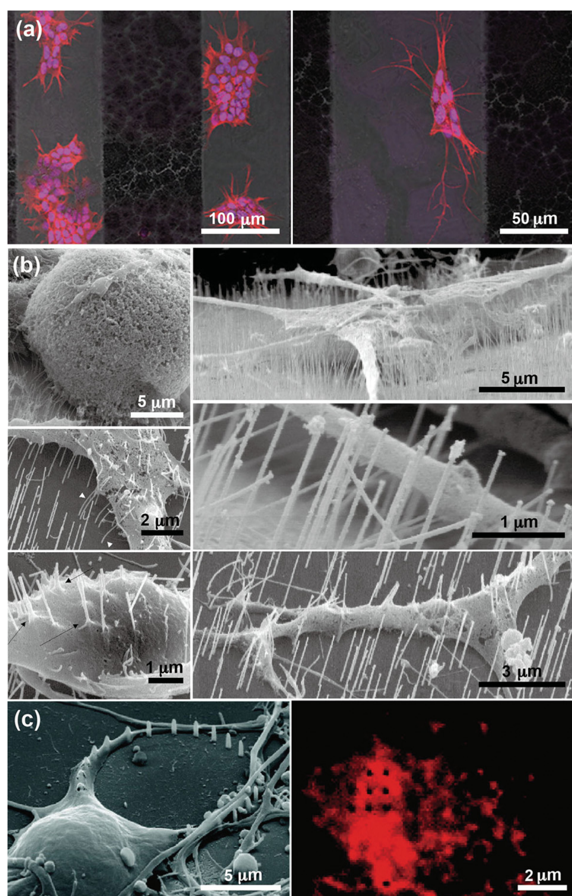

Figure 2. (a) SH-SY5Y cells were cultured on gold substrates that were composed of alternating flat or nanorough stripes. The cells selectively adhered to flat portions of the gold substrate. Reprinted with permission from [30], V. Brunetti, et al., *Proc. Natl. Acad. Sci. USA* 107, 6264 **(2010)**. © 2010, National Academy of Sciences; (b) Dissociated sensory neurons on vertically grown gallium phosphide (GaP) nanowires. Neuronal processes grew along the top of nanowires, as well as between the nanowire spaces, adhering to the sides of the structures. Both non-neuronal and neuronal cell bodies were penetrated by nanowires (black arrows indicate membrane adhesion to wires). Note the white arrows indicating nanowires bent by membrane adhesion. Reprinted with permission from [31], W. Hällström, et al., *Nano Lett.* 7, 2960 **(2007)**. © 2007, American Chemical Society; (c) SEM images show primary cortical neurons with selectively neurite adherence to noninvasive ring-forming pillars. Pillars were formed with ion-beam or e-beam induced platinum deposition. The neurites tightly wrapped around the pillars and sometimes exerted enough force to bend them. Immunostaining revealed that pillars were embedded in the cytoskeletal network of the cortical neurons. Reprinted with permission from [34], C. Xie, et al., *Nano Lett.* 10, 4020 **(2010)**. © 2010, American Chemical Society.

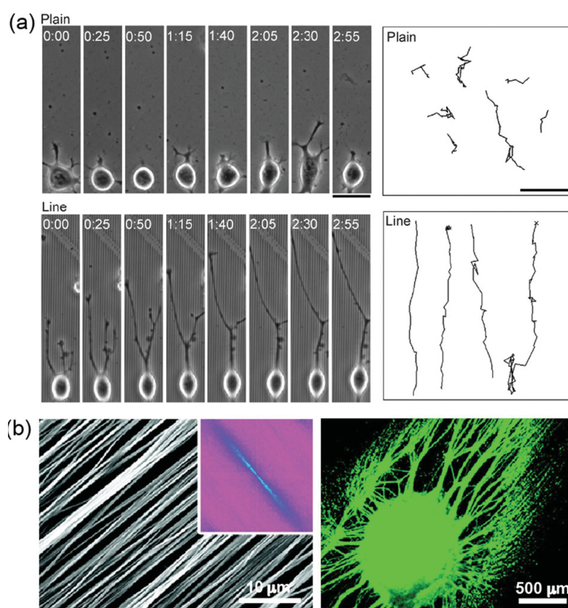

Figure 3. (a) Neurite velocity and outgrowth lifetimes were compared between cells grown on plain and line substrates. Neurite outgrowth lifetimes represent the amount of time neurites extend before retracting to the soma. Neurons grown on line substrates extended for longer periods of time and rarely retracted to the soma when compared to those on flat substrates. Neurite outgrowth velocity was also significantly increased for cells cultured on line substrates. The scale bars are 25 μm. Reprinted with permission from [38], K.-J. Jang, et al., *PLOS ONE* 5, e15966 **(2010)**. © 2010, PLOS; (b) The dendrites of DRG explants cultured on aligned PCL fibers demonstrated bipolar alignment and grew parallel to the nanofibers. The nanofibers were coated with laminin. Reprinted with permission from [55], J. Xie, et al., *ACS Nano* 3, 1151 **(2009)**. © 2009, American Chemical Society.

the grooves, coupled with an increase in neurite length as well (Fig. 3(a)). They also claimed that filopodial activities were highly responsible for the guidance effect.[38]

The Beltram group has carried out the majority of investigations using nanogroove structures with PC12 cells. Since they have reported alignment/outgrowth of differentiated PC12 cells on polystyrene nanogrooves,[39] most of their subsequent studies were implemented on cyclic olefin copolymer (COC) nanogrooves with depths of 200–300 nm and a line width of 500 nm. For example, the alignment of PC12 neurites was further considered by investigating alignment-related pathways[40] or focal adhesion maturation.[41,42] In addition, the migration of PC 12 cells has been shown to be influenced by nanogrooves.[43] The cells showed angularly restricted and slower migration characteristics compared to control cells grown on flat substrates. Recently, Tonazzini et al. reported neurite alignment of PC12 cells on the same structures, but with a quantitative destruction of groove patterns.[44] Here, nocodazole, a microtubule-depolymerizing drug, improved neurite alignment by supporting focal adhesion maturation. Similar results were observed in different cell types, including C6 glioma cells[45] and F11 neuronal cells[46] or different materials such as photo-responsive polymers (azopolymer).[47]

In addition to nanogrooves, there were more recent approaches exploiting polymer-based, electrospun nanofibers for providing nanotopographies to neural cells.

The earliest example was made by Corey et al. who demonstrated the aligned outgrowth of neurites from DRG neurons to electrospun poly-L-lactate (PLLA) nanofibers.[48] The same group also utilized these fibers for showing neurite guidance in primary motor neurons.[49,50] In further studies, electrospun fibers were functionalized with many neuro-active molecules, as exemplified by PLLA fibers decorated with basic fibroblast growth factor[51] and laminin,[52] poly-ε-caprolactone (PCL) fibers blended with collagen,[53] and PLLA-co-PCL fibers coated in carbon nanotube substrates.[54] On these decorated nanofibers, neurite guidance effect was enhanced by a synergistic effect of nanotopographical and biochemical stimuli, and some of them exhibited longer neurite development as well. PCL fibers were shown to be also effective for guiding neurite outgrowth of DRG explants (Fig. 3(b)).[55] The current neurite-guiding ability of nanofibers demonstrated by an increasing number of recent publications is receiving much attention in view of the practical application for the repair of long peripheral nerve gaps, impacting nerve regeneration fields.[56]

2.3. Enhanced Neurite Outgrowth and Developmental Acceleration

Topographical stimuli are well known to critically influence differentiation of stem cells and proliferation of other general cell types. In comparison with simple adhesion or contact guidances (migration and extension), these responses bear a higher degree of biological complexity, because they require the changes derived

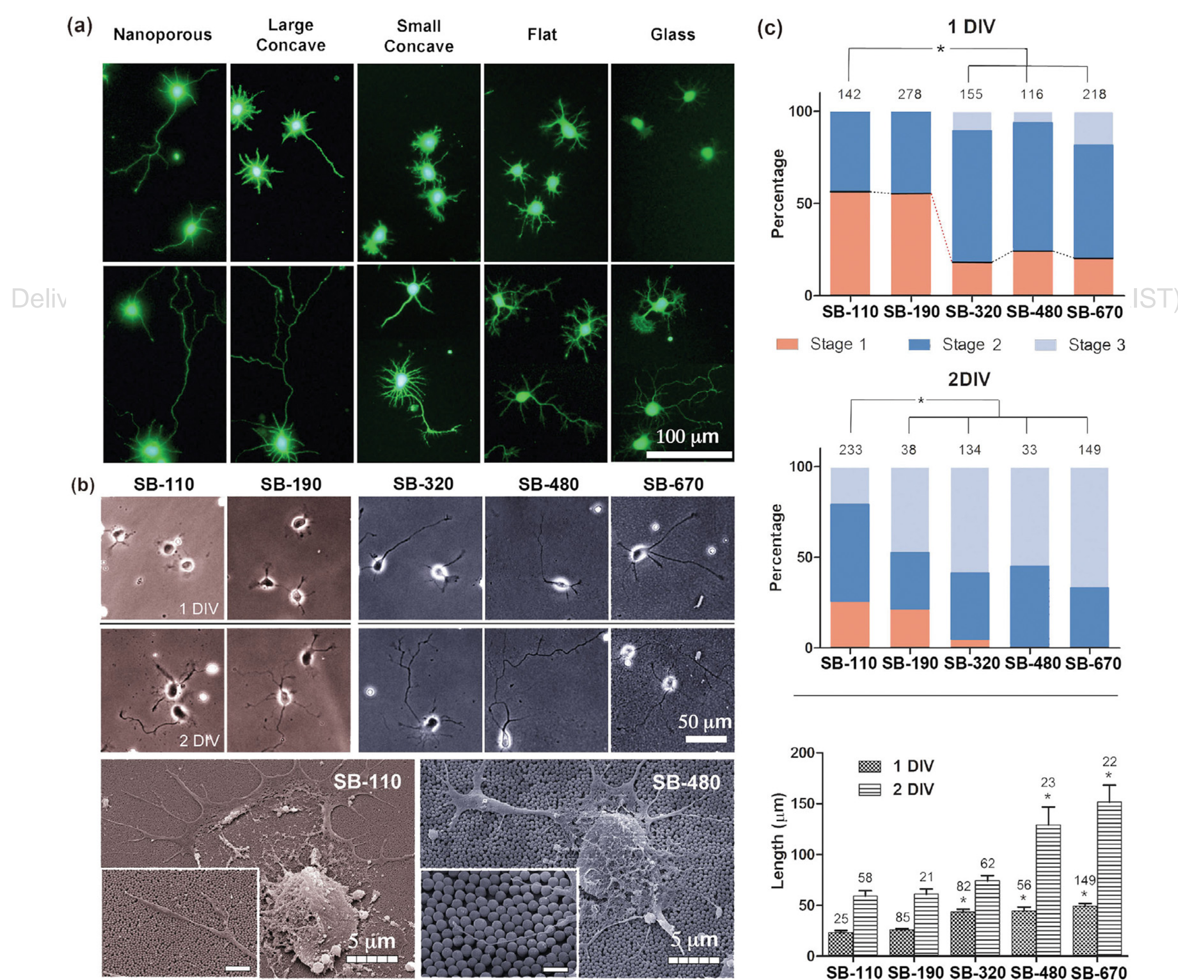

Figure 4. (a) Fluorescence micrographs of hippocampal neurons cultured on topographically different substrates. Cell morphology at 1 DIV (Top Row) and 2 DIV. Reprinted with permission from [68], K. Cho, et al., *Angew. Chem. Int. Ed.* 49, 10114 (**2010**). © 2010, Wiley-VCH; (b) Phase-contrast optical micrographs of hippocampal neurons cultured on bead-packed substrates (Top, Middle Row). SEM images of hippocampal neurons (Bottom Row). Insets: Magnified images of filopodial tips. The original gray images were rendered with a monochromatic color for the comparison between the groups. (c) Percentages of neurons in each stage at 1 DIV and 2 DIV (Top, Middle). The numbers indicate data points for the statistics. CTRL: poly-D-lysine-coated coverslips. Reprinted with permission from [69], K. Kang, et al., *Angew. Chem. Int. Ed.* 51, 2855 (**2012**). © 2012, Wiley-VCH.

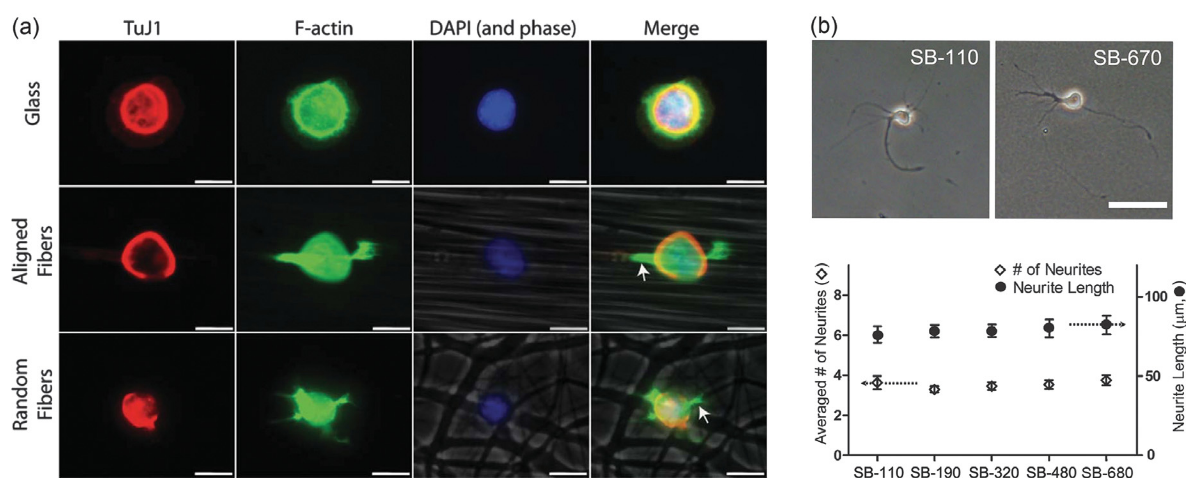

Figure 5. (a) F-actin structures were spatially restricted on nanofibers. Motor neurons were grown on glass, aligned fibers, and random fibers for 1.5 h. The fibers were imaged in phase-contrast and included in the merged images. The scale bars are 10 μm. Reprinted with permission from [50], C. C. Gertz, et al., *Dev. Neurobiol.* 70, 589 **(2010)**. © 2010, Wiley Periodicals, Inc.; (b) (Top) Phase-contrast micrographs of hippocampal neurons cultured on bead-packed substrates (SB-110 and SB-670) with the treatment of cytochalasin D. The scale bar is 50 μm. (Bottom) Quantitative analyses of the longest-neurite lengths and the average number of neurites. There were no significant differences between any pair of them for both graphs. Reprinted with permission from [69], K. Kang, et al., *Angew. Chem. Int. Ed.* 51, 2855 **(2012)**. © 2012, Wiley-VCH.

from intracellular machinery. In neural cells, such biologically complex responses appeared in the form of changes in development, since most of them neither differentiate nor proliferate. The simplest one was the longer development of neurites. Since the first demonstration of 4-hydroxynonenal (HNE)-functionalized carbon nanotubes (CNT) as a culture substrate material,[57] there have been reports regarding longer neurite development on functionalized CNT substrates. For example, neurons plated on positively charged multi-walled carbon nanotubes (MWNTs) had longer neurites than those grown on zwitterionic or negative MWNTs.[58] Single-walled carbon nanotubes (SWNTs) decorated with polyethyleneimine[59] or poly(ethylene glycol),[60] and reduced graphene oxide (rGO) substrates[61] also showed similar influences on hippocampal neurite development and PC12 cells, respectively. In addition, enhanced neurite outgrowth was broadly observed on fibers,[52–56, 62, 63] grooves,[37, 38, 40–42, 64] or nanopores[28, 30, 65, 66] along with the other responses previously mentioned.

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Recently, there has been another emerging stream of research approaching the enhanced neurite outgrowth phenomenon from an intracellular point of view. It is becoming more likely that the longer neurite development was not caused by faster neurite extension, but faster neuritogenesis. This was shown in the previously introduced work by Corey et al.[50] where the neuritogenesis and major neurite growth of primary motor neurons were significantly accelerated on PLLA electrospun fibers compared to flat surfaces, while minor neurite growth and soma spreading was restricted. Here, they introduced the *in vitro* developmental stages of neurons established by Banker et al.[67] and used the population ratios of neurons in each stage as an index for measuring the pace of neuronal development. In this way, they could prove that the neuritogenesis was indeed accelerated, not just neurite extention. The subsequent investigations by Choi et al. showed that there was a threshold roughness to elicit developmental accelerations by using anodized aluminum oxide (AAO) nanostructures (Fig. 4(a)).[68] Primary hippocampal neurons were plated on three different AAO substrates, and the results showed that nanostructures with a pitch of 400 nm had an accelerating effect on neuronal polarization, but not those with a 60 nm pitch, indicating neurons could not distinguish nanotopography with a pitch smaller than 60 nm from flat surfaces. In order to systematically investigate this nanotopography-distinguishing ability of hippocampal neurons, they had also synthesized silica beads in diameters from 100 to 700 nm and assembled them into monolayers for generating nanotopographies with different pitches (Figs. 4(b) and (c)).[69] On these substrates, the neurons cultured on smaller sized beads up to 200 nm were comparable to neurons grown on flat coverslips. But as bead sizes increased beyond 200 nm, developmental acceleration was observed for the first two days *in vitro*. Notably, this size-dependent acceleration was abolished regardless of bead diameter after treatment with cytochalasin D, an F-actin depolymerizing agent. This suggested that the F-actin based filopodial mechanisms of hippocampal neurons were highly involved in topographical sensing.

3. FUTURE PERSPECTIVES AND CONCLUSIONS

The most remarkable issue among those remaining in the field of neurons and nanotopographies is to clearly

elucidate the biological mechanism of the previously mentioned neuronal responses. As indicated by the biological complexity of observed neuronal behaviors on the nanotopographies, it is obvious that multiple intercorrelated signaling pathways accompanied with genetic regulations are involved in recognizing nanotopographies, just as how neurons recognize biomolecules and drive intracellular responses. However, the mechanism of this phenomenon is barely understood but is being elucidated by a few reports. Currently, cytoskeletal dynamics (e.g., filopodial/lamellipodial activities and microtubule dynamics) has proved to be highly responsible for the neuronal recognition of micro/nanotopographies. The physical alignment of F-actin structures to topographical features were observed by many reports (Fig. 5(a)),[27,38,50] and notably the interference in F-actin dynamics caused the complete deletion of nanotopography-distinguishing abilities (Fig. 5(b)).[69] The cytoskeletal dynamics is genetically regulated by Rho GTPases family genes (represented by Rho, Rac, and Cdc42), and this genetic regulation and the formation of focal adhesion complexes are known to play key roles in the mechanosensing of non-neural cells. Therefore, the genes related to cytoskeletal dynamics might also intensively contribute to the neuronal responses to nanotopographies, and connecting these downstream genetic regulations to the behavioral responses of neurons would be a promising direction of future research in this field. Regarding focal adhesion, it is highly required to investigate the role of integrin receptors in sensing nanotopographies, because the presence of focal adhesion complexes in neural cells is uncertain, while that of integrin receptors is not. In addition, it might be equally important to investigate the presence of mechanosensitive ion channels or mechanosensing-related G protein-coupled receptors in neural cells and their roles for recognizing topographies.

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Although we simply categorized neuronal responses into three groups (adhesion, aligned neurite extension, and developmental acceleration) on various nanotopographies, a solid principle that can generally predict the responses of neurons to the topographical types and explain the fundamentals is still required. This principle originates from the biological mechanism of how neural cells recognize topographies, and once this mechanism is thorougly understood, it is expected to have a critical impact on elucidating the development of neural systems (neuronal migration and neurite pathfinding) and also on nerve repair applications.

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