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Pioneering Effects and Enhanced Neurite Complexity of Primary Hippocampal Neurons on Hierarchical Neurotemplated Scaffolds

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In this work, the use of scaffolds is reported, templated from live neurons as an advanced culture platform for primary neurons. Hippocampal neurons cultured on neurotemplated scaffolds exhibit an affinity for templated somas, revealing a preference for micrometric structures amidst nanotopographical features. It is also reported, for the first time, that neurite complexity can be topographically controlled by increasing the density of nanometric features on neurotemplated scaffolds. Neurotemplated scaffolds are versatile, hierarchical topographies that feature biologically relevant structures, in both form and scale, and capture the true complexity of an in vivo environment. The introduction and implementation of neurotemplated scaffolds is sure to advance research in the fields of neurodevelopment, network development, and neuroregeneration.

Neuronal development in vivo is a directed process reliant on several environmental cues. Factors ranging in the form of secreted tropic molecules,[1–3] the extracellular matrix,[4–10] or even other cells[11–13] all play a critical role in guiding neurite outgrowth throughout the body. The presence and position of other neurons, in particular, has been shown to act as structural landmarks to newly developing axons in search of their target.[11–13] While "pioneer neurons" are not a specialized type of neuron, their presence and positioning acts as a physical checkpoint for the efficient development of an appropriately connected neuronal network.

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Many studies have been conducted to characterize the biological importance of physical cues in neuronal development. Recent years, especially, have witnessed a rapidly growing interest in the topographical responses of neurons, including neuronal stem cells, for potential applications in developmental neurobiology and neuroregenerative medicine.[14–16] A variety of structures have since been used as platforms for neuron culture, with different physical features influencing different aspects of neuronal behavior. For instance, anisotropic topographies, such as grooves,[17,18] pillars,[19,20] and fibers[21,22] have been shown to affect the orientation of neurite outgrowth and guide neurites along a desired direction. Nanometric topographies have also been extensively utilized; nanowells,[23] nanobeads,[24,25] nanopillars,[20] and nanowires[26,27] alike have accelerated neurite outgrowth, and in the case of the latter, even induced an in vivo-like pathway of development.

Despite the wide variety of substrates that have been used in topographical studies, most features have still been considered simplified representations of the complex cellular environment in vivo, in both form and scale. For instance, a developing neuron in vivo would encounter a variety of structures, such as somas, which are tens of micrometers in diameter, and the submicrometer fiber-like neurites of other neurons. These diverse physiological cues present in the natural environment of a neuron strongly suggest that hierarchical topographies, featuring both micro- and nanometric structures simultaneously, should be a critical component of neuron culture platforms. The synergistic value of hierarchical topographies has been observed in several other cell types, such as embryonic stem cells,[28] myoblasts,[29] osteogenic precursors,[30] or neural stem cells,[31] yet most topographical substrates used in primary neuron research are typically singular in form, scale, or array, which fail to capture the complexity of the true neuronal environment.

Cell-templated scaffold has demonstrated a great potential in a diverse range of applications, such as cell sorting and targeting, and diagnostics, because it could replicate the surface topography of sacrificed cells.[32,33] In this work, we fabricated topographies that possessed hierarchical micro- and nanoscale structures templated from live neurons as a platform to observe

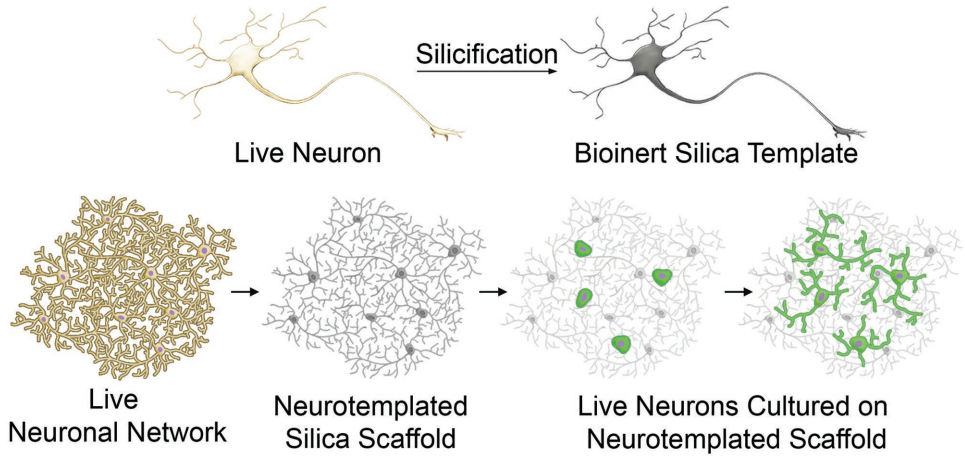

Figure 1. Schematic representation of neurotemplated scaffold fabrication.

the effects of a structurally accurate neuronal network on neuron development (**Figure 1**). Neurotemplated scaffolds (NTSs) were created via the silicification of living neurons, creating bioinert structures that preserved the morphology and scale of a naturally formed neuronal network. We found that neurites possessed a high affinity for templated somas rather than templated neurites, offering insight to the scalar preferences a developing neuron may exhibit in vivo. We also report for the first time that neurite complexity could be topographically controlled depending on the nanometric density of the underlying scaffold. We believe that neurotemplated scaffolds as a hierarchical topography that accurately represent the intricate micro- and nanometric structures of real neurons and neuronal networks, are sure to be an important advancement for platforms in the study of neuron development, neuroregeneration, and neuronal network formation.

Neuronal networks are complex webs composed of countless neurites and cell bodies, and possess features that span a spectrum of dimensions. In order to accurately capture such intricacies, living neurons were cultured, fixed, and templated to reflect the physical sizes and structures of a naturally formed neuronal network, resulting in hierarchical neurotemplated scaffolds. Primary cortical neurons, selected for their superior yield, were seeded on flat, glass coverslips at a density of 1000 cells mm^{-2}, and were cultured for 7 days in vitro (DIV) to form a mature neuronal network. The cultured cells were silicified and calcinated[34] to form biologically inert, neurotemplated topographies. Scanning electron microscopy (SEM) images showed that cellular structures were well preserved from the network and cell bodies to neurites and filopodia (**Figure 2**a). Samples were coated with poly-D-lysine, a positively charged neuroadhesive polymer, and prepared for cell culture. Neurotemplated scaffolds displayed no detrimental effects on neuron viability when compared with flat, glass coverslips (Figure S1, Supporting Information).

As with any physiological process, axon pathfinding is not reliant on solely biomolecular or physical stimuli, but rather an interplay between both factors that is challenging to disentangle. However, the bioinert nature of the neurotemplated silica scaffolds allowed us to isolate physical stimuli when characterizing the effects of an established neuronal network on newly developing neurons. Primary hippocampal neurons were cultured on neurotemplated scaffolds and compared with neurons cultured on flat, glass coverslips as a control (Figure 2b). Because both glass coverslips and neurotemplated scaffolds were composed of bioinert silica, it was supposed that only topographies of substrates could affect neuronal behaviors. We explored the potential pioneering effects and guidance capabilities of neurotemplated scaffolds on developing neurons by characterizing neurite interaction with templated structures, and aimed to gain insight into structural preferences neurons may exhibit when simultaneously presented with micro- and nanoscaled features of varying geometries.

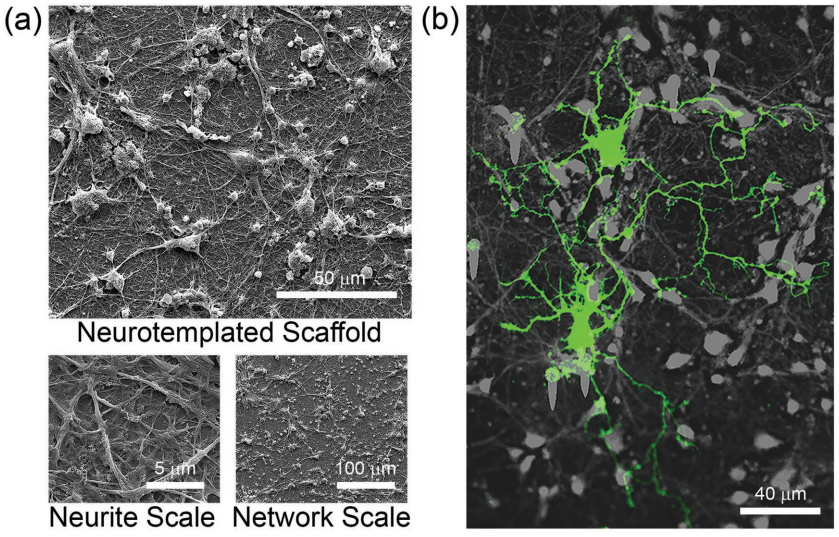

Figure 2. a) SEM images of neurotemplated scaffolds. Physical features are preserved across relevant scales. b) Confocal laser-scanning microscopy (CLSM) image of primary hippocampal neurons (green) cultured on neurotemplated scaffolds (gray).

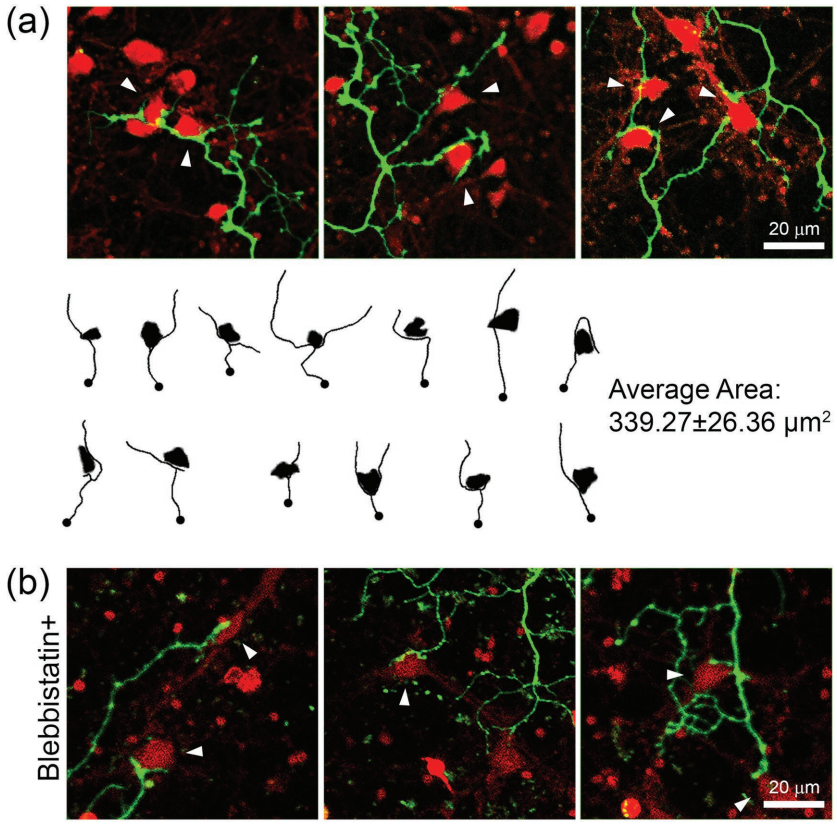

Figure 3. a) CLSM images of hippocampal neurites on bioinert neurotemplated scaffolds. Neurites (green) display high affinity for templated somas (red) amidst the "topographical noise" of templated nanostructures. White arrows indicate neurites wrapping around templated somas. b) CLSM images of blebbistatin-treated (blebbistatin+) hippocampal neurites on bioinert neurotemplated scaffolds. White arrows indicate neurites wrapping around templated somas.

We found that neurons cultured on neurotemplated scaffolds were largely unaffected, directionally, by the underlying templated neurites. Instead, neurites showed a high affinity for templated somas, exhibiting their preference by wrapping themselves around the rounded structures before continuing to elongate (**Figure 3**a). The contacted somas possessed an average area of 339.27 ± 26.36 μm^2. The recognition of other cells in vivo has been suggested to result from a synergistic combination of physical recognition, tropic factors, and contact-mediated intercellular exchanges.[35] Our results indicated that while biomolecular cues may serve to guide neurite outgrowth more efficiently or confirm the correct course, physical structure alone may be enough for neurites in vivo to distinguish somas amidst other physiological components, even without the presence of cell-specific receptors.

Additionally, while previous reports show neurites recognizing features as small as 0.79 μm^2 or even less,[36,37] the smallest observed templated-soma that neurites displayed an affinity for, in this work, was 185.22 μm^2. We presumed that the surrounding presence of innumerable nanostructures (in the form of templated neurites and filopodia) created "topographical noise," which significantly elevated the minimum required size for recognition by neurites. This possibility was corroborated by the observation that the neurites of neurons cultured on neurotemplated scaffolds with significantly decreased nanotopographical density interacted with not only templated somas but also templated neurites (Figure S2, Supporting Information). When considering the high density of neurons in the brain, we believed that the selective affinity for larger micrometric structures on hierarchical topographies better encapsulated the natural preferences of neurons in vivo. Further characterization of neurotemplated scaffolds also revealed that a templated soma is smoother than its surroundings, with an R_a of ≈50 nm compared with ≈140 nm, respectively, suggesting that roughness may also be a distinguishing factor during recognition (Figure S3, Supporting Information).[38]

We conducted biochemical inhibition assays to explore the intracellular mechanisms involved in the recognition of templated somas. Neurite outgrowth on topographies has been reported to be cytoskeleton dependent, largely relying on actin dynamics. For instance, actomyosin contraction had also been shown to play a critical role in topographical recognition, as neurons treated with blebbistatin did not respond to nanotopographical cues.[24] We, however, discovered that, blebbistatin-treated neurons were still able to recognize templated somas (Figure 3b). This suggested that recognition and subsequent adhesion to target structures on hierarchical topographies are not critically dependent on actomyosin contraction and that, interestingly, all topographically induced responses, such as neurite elongation or axon pathfinding, are not operated by the same intracellular mechanism.

In addition to providing insight into the structural interactions and affinities neurons may present in vivo, neurotemplated scaffolds also allowed us to characterize the intracellular effects of hierarchical topographies, manifested via changes in neuronal development and morphology. For instance, we found that neurons cultured on neurotemplated scaffolds possessed significantly longer neurites compared to the control (**Figure 4**a). At 1 DIV the longest neurites of neurons on neurotemplated scaffolds had an average length of 66.54 ± 3.29 μm compared to 33.30 ± 1.66 μm on glass coverslips (Figure 4b). At 2 DIV, the longest neurites of neurons on neurotemplated scaffolds were again about twice as long as those on flat coverslips. Additionally, neurons cultured on neurotemplated scaffolds displayed an accelerated rate of development (Figure 4c). At 1 DIV, 75.3% of neurons on neurotemplated scaffolds were in stage 2 (neurite formation), compared with 37.9% on the coverslips. At 2 DIV, a large majority of neurons (73.6%) cultured on neurotemplated scaffolds were now at stage 3 (major neurite elongation), compared with 16.5% of neurons on flat coverslips. The higher population of stage 2 neurons on neurotemplated scaffolds at 1 DIV indicated that the hierarchical topography stimulated the earlier occurrence of neuritogenesis in developing neurons, and a greater population

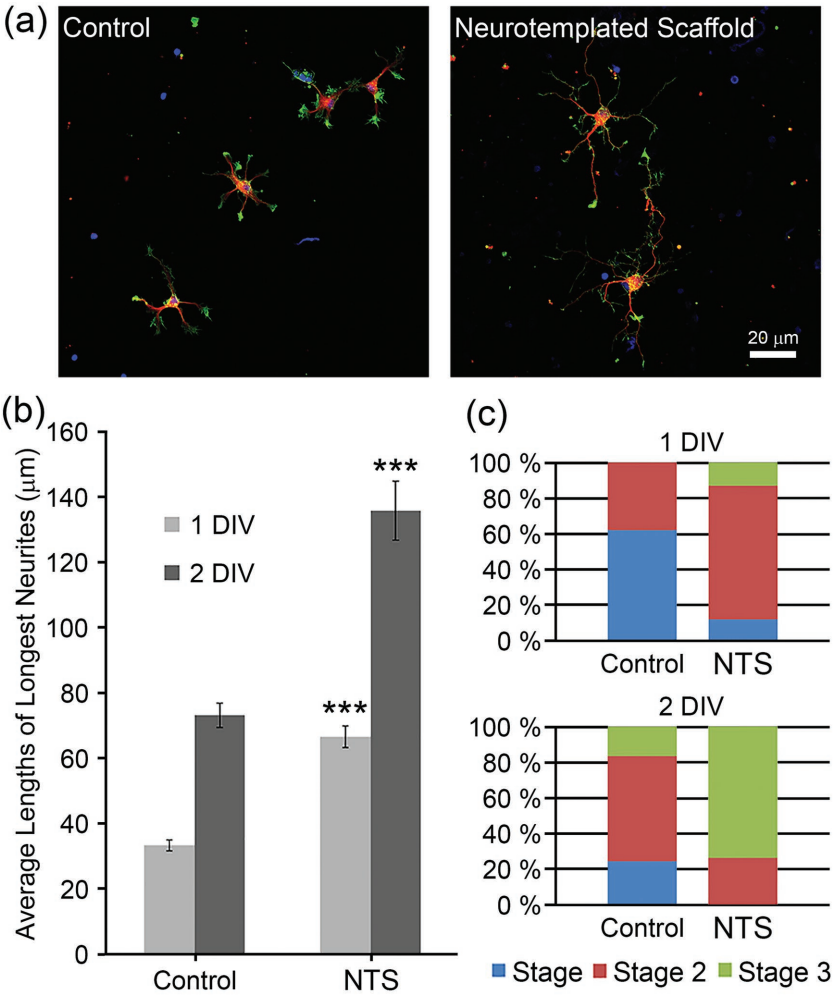

Figure 4. a) CLSM images of neurons cultured on flat coverslips and neurotemplated scaffolds. F-actin (green), microtubules (red), and nuclei (blue) were fluorescently labeled. b,c) Average lengths of longest neurites (mean ± standard error) and developmental stages of neurons on neurotemplated scaffolds. NTS: Neurotemplated scaffold. All of the results were analyzed by one-way analysis of variance (ANOVA) with post-hoc Tukey honestly significant difference (HSD) test ($n = 105, 109, 124, 115$ for control at 1 DIV, control at 2 DIV, NTS at 1 DIV, and NTS at 2 DIV, respectively; $^{***}P < 0.001$).

of stage 3 neurons at 2 DIV indicated that determination of the axon occurred more rapidly as well.

Neurotemplated scaffolds were also highly versatile topographical platforms, as any variations in the physical structure of the original templated neuron cultures were translated to the final silica substrates. We created three different neurotemplated scaffolds with low, mid, and high topographical densities (*l*-NTS, *m*-NTS, and *h*-NTS, respectively) by adjusting the seeding density of template neurons (100 cells mm^{-2} for *l*-NTS, 500 cells mm^{-2} for *m*-NTS, and 1000 cells mm^{-2} for *h*-NTS) to characterize the effects underlying neurotemplate density (**Figure 5**a). SEM images showed that *l*-NTS mainly featured micrometric neurite structures, with much of the underlying, flat substrate exposed. The *m*-NTS substrates possessed not only micrometric neurites, but also a greater amount of nanometric neurite branching, with some of the underlying flat substrate exposed. The most topographically dense substrate, *h*-NTS, also consisted of both micrometric and nanometric neurites, which completely covered the entire surface so that virtually none of the flat areas were visible. Quantification of the scaffolds via atomic force microscopy (AFM) showed that the surface roughness of *l*-NTS, *m*-NTS, and *h*-NTS were about 88, 107, and 140 nm, respectively.

Nanometric topographies have been known to affect several facets of neuronal behavior, including neurite length, directionality, or even pathways of development;[39,40] yet one function that has not been reported is control over neuron complexity using nanoscale features. Neurite complexity is a critical characteristic of neurons, as these values determine the amount of synaptic connections that a single neuron can make and ultimately increase the potential signaling complexity and efficiency of an interconnected neuronal network.[41] We discovered, via sholl analysis,[42] that nanoscaled topographical density was directly proportional to neurite complexity, with neurite complexity being highest on *h*-NTS and lowest on *l*-NTS (Figure 5b and Figure S4, Supporting Information). In other words, nanometric structures increased the number of neurites, degree of neurite branching, and dendritic length. Despite the variations in nanotopographical density, we found that at 2 DIV there was no statistically significant difference in neurite length between the three different topographies (Figure 5c). Neurons on neurotemplated scaffolds most likely reached an upper threshold for their accelerated development.[24,25] A significant decrease in topographical density was required to lower the degree of developmental acceleration (Figure S5, Supporting Information). These results indicated while *l*-NTS substrates were relatively sparse of nanometric features, micrometric topography was sufficient to maximally enhance major neurite elongation[24]; the additional presence of nanometric structures did not necessarily increase the threshold of enhancement.

Taking into account the physical variations between the three scaffolds and their respective effects on neuron morphology, we suggested that micrometric and nanometric features on hierarchical topographies direct different aspects of neurite development. Although precise underlying mechanism of neuronal responses to topographical cues is still unrevealed, a lot of studies have pointed out that focal adhesion (FA) formation, integrin activation, cytoskeletal rearrangement, and ROCK (Rho-associated protein kinase) signaling are mainly contributed to topographical regulation of neuronal maturation.[15,24] Neurite formation and branching is initiated by the reorganization and protrusion of actin and microtubules, and is closely correlated to local formations of FA complexes.[41,43] Since nanotopographies have been shown to affect the orientation and localization of FAs,[18] which are also nanometric in size,

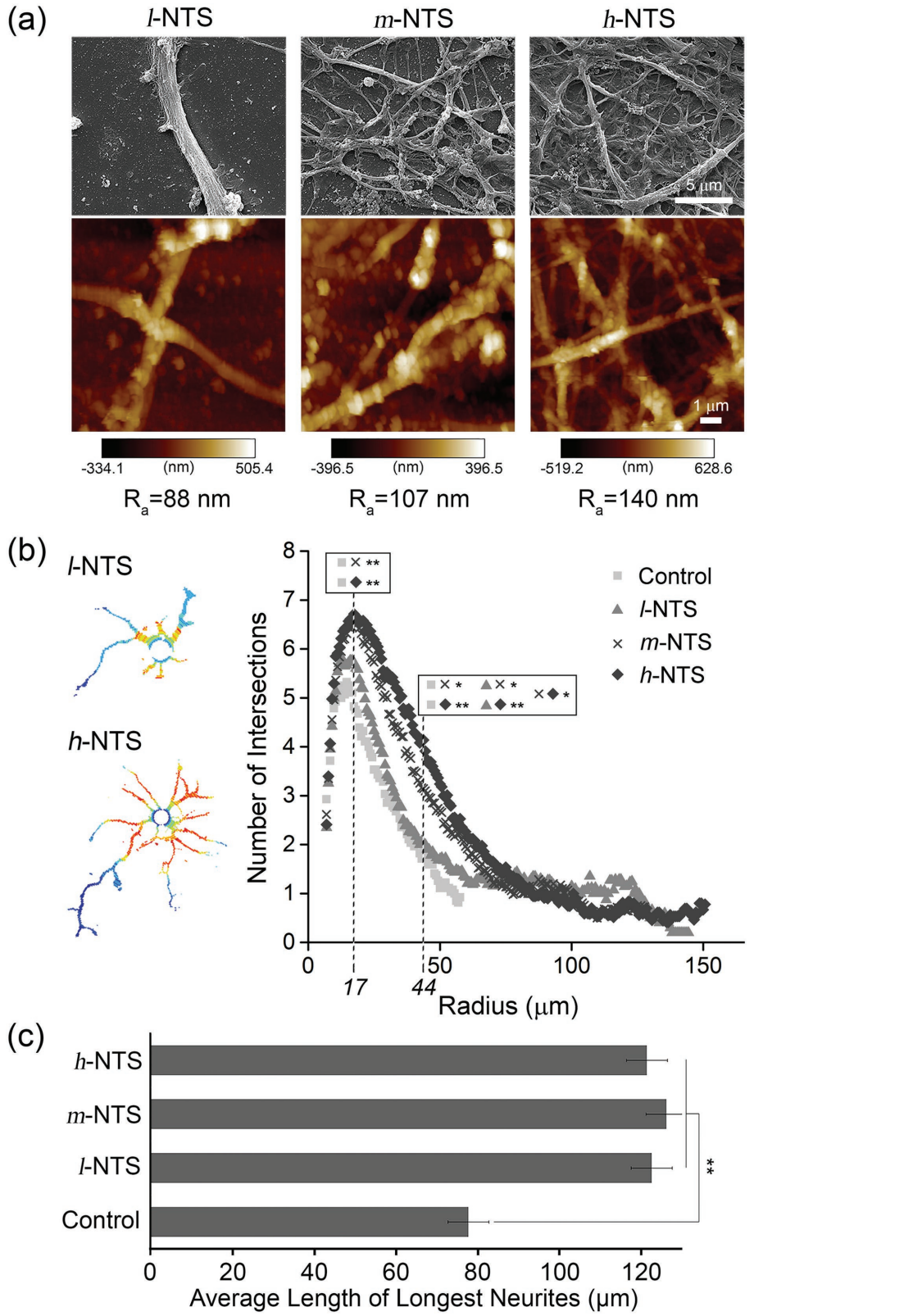

Figure 5. a) SEM and AFM images of *l*-NTS, *m*-NTS, and *h*-NTS substrates. b) Sholl analysis of neurons on neurotemplated scaffolds. As a representative, the statistical analysis was performed with radii of 17 and 44 μm. c) Average lengths (mean ± standard error) of longest neurites on neurotemplated scaffolds. All of the results were analyzed by one-way ANOVA with post-hoc Tukey HSD test (b: $n = 48$, 43, 63, and 58 for control, *l*-NTS, *m*-NTS, *h*-NTS, respectively; c: $n = 118$, 117, 102, and 152 for control, *l*-NTS, *m*-NTS, and *h*-NTS, respectively; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

it is conceivable that templated filopodia on NTS substrates act as distinct adhesion points for FAs to form, resulting in several initiation sites for neurite branches to form. Micrometric features alone, on the other hand, were too large to selectively influence interfacial adhesions. This scale-dependent division of effects on neurite morphology further emphasized the importance of hierarchical micro/nano topographies in the study of neuron development and network formation.

In summary, we have shown that not only is there a scale-dependent division of influence of neuron behavior, but also extracellular recognition of topographical features and intracellular reaction to those features could both be modulated by the ratio between micro- and nanotopographies. Neurites on neurotemplated scaffolds exhibited an affinity for micrometric somas amidst the topographical noise of nanometric neurites. Neurons also exhibited enhanced neurite elongation and accelerated development independent of the nanometric density of the scaffolds. However, neuron complexity was discovered to be directly proportional to the presence of nanometric neurite branches and filopodia. These findings underline the importance of implementing hierarchical topographies in neuronal research, as well as the advantages of neurotemplated scaffolds for studies in neuronal development, neuroregeneration, and neural prostheses.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

axonal pathfinding, hierarchical topographies, neurite complexity, neurotemplated scaffolds, pioneer neurons

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