Distance Dependence of Neuronal Growth on Nanopatterned Gold Surfaces

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Understanding network development in the brain is of tremendous fundamental importance, but it is immensely challenging because of the complexity of both its architecture and function. The mechanisms of axonal navigation to target regions and the specific interactions with guidance factors such as membrane-bound proteins, chemical gradients, mechanical guidance cues, etc., are largely unknown. A current limitation for the study of neural network formation is the ability to control precisely the connectivity of small groups of neurons. A first step in designing such networks is to understand the “rules” central nervous system (CNS) neurons use to form functional connections with one another. Here we begin to delineate novel rules for growth and connectivity of small numbers of neurons patterned on Au substrates in simplified geometries. These studies yield new insights into the mechanisms determining the organizational features present in intact systems. We use a previously reported atomic force microscopy (AFM) nanolithography method to control precisely the location and growth of neurons on these surfaces. By examining a series of systems with different geometrical parameters, we quantitatively and systematically analyze how neuronal growth depends on these parameters.

Introduction

The basic working unit of the brain is the neuron, a specialized cell consisting of a cell body, plus two types of processes: a single long threadlike axon that transmits electrical impulses and shorter, thicker dendrites that receive messages from the axons of other neurons. In the developing brain chemical and geometrical cues are an essential source of information used by these processes when wiring up the nervous system. Over the past few years, many of the molecular cues and the signaling pathways through which they operate have been identified using traditional biological techniques. However, our understanding of the mechanisms by which the guidance factors control the path that growing processes follow to reach their targets remains qualitative. The main problem lies in the complex and highly structured control of neuronal matter in the brain, which has proven immensely challenging to study in vivo. Furthermore, controlled in vitro studies of neuronal growth using classical neuron-plating techniques are difficult to perform because of the random organization of neurons, even in low-density samples. An alternative approach used to create highly organized neuron networks is to culture neurons on substrates with appropriately patterned adhesion and guidance materials. The principle behind these methods is to pattern both cytophilic (cell-attractive) and cytophobic (cell-repulsive) chemical and geometrical cues on the same surface and to use these patterns to confine both the position of the soma and the elongation of the neurites. For example, it has been shown that by engineering patterns of proteins on two-dimensional surfaces one can create simpler, artificial neural networks for manipulation and study. Many micropatterning techniques have been developed in order to define and manipulate the environment of single cells, such as UV and plasma etching techniques, microcontact printing, dip-pen microlithography, dip-pen printing, and laser ablation. In all of these methods, the substrate is flat and the patterning is performed in a manner that limits the degree of organization one can achieve in neuronal cultures.

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nanolithography, microfluidic patterning, and carbon-nanotube patterned substrates. Researchers have used these techniques to create simple neuron networks such as lines and grids and to selectively guide axonal growth on micropatterned molecules. It has recently been shown that, because the adhesion sites of the cell are in the range 5–200 nm, the cell adhesion is strongly influenced by nanoscale topographical and biochemical features. Therefore, a successful neuron patterning technique should combine several capabilities such as high patterning resolution, easy alignment of the cell patterns to other features already present on the substrate (such as electrodes or microfluidic channels), and the ability to print multiple different biomolecular cues on the same surface. At present, microcontact printing using various substrates (polydimethylsiloxane or PDMS, glass, silicon dioxide, etc.) is the most commonly used technique to selectively pattern cell-adhesive and cell-repulsive molecules on a micrometer scale. However, the use of microcontact printing is still challenging for certain applications such as creating submicrometer patterns, substrate gradients of biomolecular cues, or surface immobilization of biologically active molecules.

We have recently introduced a new approach for controlling the adhesion, growth, and interconnectivity of cortical neurons on Au surfaces. Instead of micropatterning molecules that facilitate neural growth on glass or plastic, we first create a very inhibitory substrate (formed from undecanethiol triethyleneglycol molecule self-assembled on Au, simply referred to as PEG/Au). The substrate could then be removed at well-defined locations via AFM nanolithography (nanoshaving). Next, the patterned surface is exposed to solutions of growth-promoting molecules (growth factors, extracellular matrix proteins, etc.) that adhere with high yield to the nanoshaved areas, while the bare gold surface is exposed. The patterns are designed such that the neurons preferentially adhere and grow on the growth-promoting patterns, an approach that dramatically reduces neuronal adhesion in all areas other than where the inhibitory substrate is removed. We have demonstrated that this method allows us to control adhesion factors that can be used to influence the growth and development of neuronal assemblages in simple controlled geometries.

We have shown that this method provides several advantages for this type of work: high degree of control over the location and shape of protein patterns; the proteins are likely to retain their biological activity (the procedure is carried out in aqueous solutions and does not require drying steps); the technique allows creation of nanoscale (down to several tens of nanometers) biomolecular patterns on substrates. The major limitation of this technique is that, compared to other micropatterning approaches such as microcontact printing, it has a lower throughput and therefore is mainly applicable for creating patterns with a relatively low number of neurons (10–100). We note, however, that in principle it is possible to use dedicated AFMs with special nanolithography software to create large numbers of biomolecular patterns on substrates using this approach.

In this paper we show that neurite growth is strongly promoted by narrow (1 μm width) lines of patterned proteins. In addition, we show that the growth of neuronal processes on well-controlled patterned substrates depends systematically and nontrivially on the geometric patterns and that under proper conditions neurite growth on the unpatterned, cell-resistant substrate can also be induced. This situation resembles some aspects of in vivo conditions, where neurons grow frequently along pathways of permissive substances that are flanked by extensive areas of cell resistant/inhibitory molecules. By quantifying neuronal growth on these substrates, we obtain insights into the intrinsic and extrinsic factors governing process outgrowth and connectivity. In particular, we perform an experimental investigation of the dependence of process outgrowth on the distance between adjacent pairs of neuron cell bodies and on the length/shape of molecular cues (protein patterns). For this study we use a total number of 20 different types of patterns, with a total of 100 different samples.

### Experimental Methods

#### a. Substrate and SAM Preparation

It is well-known that long-chain PEG molecules self-assembled on Au surfaces, forming a self-assembled monolayer (SAM), which is very efficient for resisting both protein and cell adhesion. Moreover, annealed Au provides a flat 2D surface, which is extremely effective for protein immobilization. The Au surfaces are prepared by e-beam evaporation on mica substrates in a vacuum chamber (Denton Vacuum, model DV 502-A). The mica (clear ruby muscovite mica, New York Corp.) is preheated to 350 °C before deposition. After evaporation, Au films (50 nm thick) are annealed at 360 °C under vacuum for 30 min, cooled to room temperature, and immersed into 0.1 mM undecanethiol triethyleneglycol (HSC11-EG3), simply referred to as PEG (Prochimia, 98% purity), solution for 48 h, so that a compact monolayer forms on the Au (111) surface. Before characterization by AFM, the sample was rinsed for 2 min with pure ethanol and dried by nitrogen flow.

#### b. AFM Lithography

AFM lithography and topographical imaging studies were performed using a Digital Instruments MultiMode AFM (Santa Barbara, CA) with a Nanoscope IIIa controller and Type J scanner (Digital Instruments). Most AFM experiments were carried out in contact mode, in a liquid cell kept at room temperature and commonly available. Van der Pauw cantilevers (NPS, Veeco Instruments). Protein patterning was done using an AFM nanolithography procedure called nanoshaving (Figure 1).

In this procedure, an AFM tip is first scanned, at low force, to image the surface topography and select a certain region on the PEG/Au substrate. Second, the selected micrometer-size area of PEG is shaved away by the AFM tip under high force loads (~100 N), while the SAM is in contact with a pure solvent (ethanol) (Figure 1A). The PEG molecules released into the solution are then captured by the SAM. The AFM tip is then scanned at high force loads (~350 N) over the desired area, while the tip is kept in contact with the SAM. The PEG molecules released into the solution are then captured by the SAM. The AFM tip is then scanned at high force loads (~350 N) over the desired area, while the tip is kept in contact with the SAM.
by the AFM tip into the ethanol solution have a very low concentration and therefore have a negligible probability of returning to the Au surface. Finally, the ethanol solution is replaced with a buffer solution containing the protein of interest (poly-lysine, or PDL), and the sample is soaked in this solution for 45 min (Figure 1B). Since the PEG is very efficient in resisting protein adsorption,40–51 the proteins adhere to the substrate only on the exposed Au regions where the SAM was removed by nanoshaving.56 The protein patterns were validated by postshaving AFM imaging.36

**Results and Discussion**

**a. Influence of Pattern Geometry.** We demonstrate that PDL lines strongly influence process growth, in the sense that processes favor growth on the patterned PDL regardless of changes in direction. Figure 2 shows schematics of simple patterns as well as optical micrographs of neuronal growth after 5 days of incubation. In Figure 2A there is a neuron located on each of the PDL squares (growth squares). A third neuron adheres close to the top growth square, outside the patterned area. As reported previously, a small percentage (10–15%) of the neurons adhere to the substrate in unpatterned locations with defective PEG.36 Figure 2A shows that the PDL line emanating from the lower square guides process growth, as is indicated by the change in direction as the process follows the bend in the line, instead of continuing to grow straight toward the neuron at the top square. We have reported similar data for various distances between cells, including separations <45 μm between the PDL line bending point and the second neuron.20,38 Figure 2B shows an example of an isolated neuron, with the cell body adhering to the PDL growth square and four processes growing along the PDL lines. In contrast, Figure 2C shows that there are no processes growing out of the cell body for neurons adhering to isolated PDL squares without PEG. In some cases of isolated neurons, possible remnants of short processes are visible (Figure 2D), but we do not have time-lapse imaging data, we cannot distinguish at this point if these are remnants of real process growth or of the cell body after the fixation procedure. In all cases of isolated neurons we do not observe any long process growth comparable to case presented in Figure 2B, for example. These and similar results obtained on ~50 samples28,29 show that processes strongly favor growth on patterned PDL, regardless of changes in direction. We therefore conclude that the presence of protein lines and/or other cells positively affects the viability of neurons grown on these samples. Nonetheless, as we discuss below and as expected, process growth is far more complicated than this simple picture suggests, involving several more factors than simple interaction with a patterned substrate. The key advantage of the patterned substrate is that it provides an additional and controllable driving force.

**b. Comparison between Process Growth on Patterns with PDL Lines vs Patterns without PDL Lines.** Figures 3 and 4 show that neurons can grow over the normally inhibitory PEG substrate if (a) the neurons are patterned close to each other (Figure 3) or (b) if neuronal cell bodies are positioned farther apart but thin PDL lines guide neurites toward one another (Figure 4). Data obtained on multiple (20) samples similar to the ones shown in Figure 3 demonstrate that if neurons are patterned on 10 μm square patterns of PDL that are positioned within 35 ± 5 μm of one another (edge-edge distance D), they will extend several processes off the squares onto the PEG.
and ultimately fasciculate with other neurons patterned on PDL squares in the near vicinity. We also find that neurons send out shorter and fewer processes as the separation distance between the cell bodies is increased (Figure 3).

Surprisingly, if neurons are patterned on squares that are 100 μm apart but have short (15 μm) lines pointed toward each square, the neurons can bridge the 70 μm of PEG substrate separating the two PDL line segments (Figure 4B). However, if this distance is longer than 70 μm, the neurons cannot bridge the gap (Figure 4C, D). This result is different from the data on patterns without PDL lines (Figure 3), where neurons fail to grow processes that connect if they are separated by more than 35 μm.

All the images shown in Figure 3 and 4 are taken for neurons grown for 5 days. Previous reports show that at low densities (1000–3000 cells/cm², similar to the cultures presented here) cortical axons average 250–300 μm in length after 2 days in culture, and continue to grow and branch as long as they are in culture. However, the process length for the samples grown on PDL/PEG/Au are much shorter (Figure 5), and images taken on samples kept for longer incubation times (up to 2 weeks) show no significant difference in the length or number of processes. These results strongly suggest that the chance of connection between axons does not increase for longer incubation time. The questions of how fast the processes grow in different geometries and what is the minimum time required to establish a connection are currently under investigation using time-lapse microscopy experiments.

c. Process Growth and Connectivity vs Separation between Neurons. To analyze how neuronal growth depends on the distance between pairs of neurons, we show in Figure 5A plots of the length $L$ of the longest process grown on PEG in each neuron pair as a function of the separation between sources (see below) for both types of patterns shown in Figures 3 and 4. Similarly, Figure 5B shows the total number of processes grown by neurons on PEG as a function of the separation between sources.
as the distance $D$ between the edges of the growth squares for the case when there are no PDL lines and the distance $d$ between the edges of the PDL lines for the other type of patterns. Thus, parts A and B of Figure 5 show the actual length of the longest process and the total number of processes, respectively, grown over the inhibitory PEG substrate for patterns with no PDL lines (blue dots) and PDL lines (red dots).

The blue data points in Figure 5A show that the length $L$ of the longest process grown over the PEG substrate increases with separation distance $D$ up to a maximum value $L \sim 20 \mu m$ reached when $D$ has a critical value: $D_c = 35 \mu m$. It is important to emphasize that $D_c$ also represents the maximum distance the cells can bridge and make a connection in the absence of PDL lines. Above $D_c$, the process length decreases rapidly with increasing separation. There are no processes growing out from the cell bodies for $D > 100 \mu m$, which is consistent with our previous observations that isolated neurons on PEG do not form neurites (Figure 2C and ref 36).

Similarly, the red data points in Figure 5A show that for growth squares having PDL lines patterned in between (see Figure 4) the length of the longest process $L$ increases with increased separation $d$ between the edges of the PDL lines, reaches a maximum at $d = d_c = 70 \mu m$, and then decreases rapidly. In this case, processes grow on PDL lines even for large separations between cell bodies (e.g., $D = 170 \mu m$, see also Figure 4D) consistent with similar observations of growth on isolated neurons when PDL lines are present (Figure 2B). However, there are no significant processes growing out on the PEG for $d > 110 \mu m$ (similar to the case $D > 100 \mu m$ for no PDL lines, see above). This result suggests that the separation $d$ between the edges of the PDL lines, rather than the separation $D$ between the edges of the squares, is the relevant length scale for this type of pattern (similar observations can be made for the total number of processes, as shown in Figure 5B).

We emphasize that since in the experiments presented here the neurons are not stained for postsynaptic proteins, the process length can be measured unambiguously and with high accuracy only when the neurons do not fasciculate ($D > D_c$ for blue points in Figure 5A and $d > d_c$ for the red points in Figure 5A). Only data for neurons that do not fasciculate will be used for analysis in the next section. For patterns with PDL lines, two neurons make at least one connection when $d \leq d_c$, as shown in Figure 4A-B. We note that for all cases when neurons form connections the data shows that the length of the process growing on PEG increases with increasing separation (see Figure 4A-B, for example). For separations $d$ within this range, the longest processes always grow along the direction promoted by the PDL lines.

The two graphs in Figure 5A (blue and red points) have a similar shape and show similar behavior in the limit of large separation distances $D$ or $d$. However, there is an important difference between the two cases: neurons can bridge over 70 $\mu m$ of PEG substrate that separate the two PDL lines compared to 35 $\mu m$ (at most) for the case without the PDL lines.

The blue data points in Figure 5B show that the total number of processes $N$ for a neuron pair decreases monotonically with increasing separation distance $D$ between the pairs for patterns without PDL lines (Figure 3). In contrast, the red data points in Figure 5B show that patterns with PDL lines yield a total number of processes $N$ that is independent of the separation distance $d$ for $d \geq 70 \mu m$ (that is, when the neurons bridge the PEG gap and form a connection) and which decreases rapidly as the separation distance is increased above this threshold. Combined with Figure 5A, these plots show that neurons in close proximity actively grow many, long processes and that beyond $\sim 100 \mu m$ separation (defined by the relevant distance $D$ or $d$, see above) very little process outgrowth occurs.

### d. Diffusion Model for Neuronal Growth on PDL Patterns

We have cultured small numbers of neurons on substrates with patterned proteins in which the geometry is varied systematically. As previously reported,36 we find that cell bodies adhere with high probability on regions with patterned PDL and with very low probability on regions not specifically patterned with PDL and thus covered with PEG.36 Reference 36 gives specific guidelines for AFM patterning procedures that minimize the accidental incorporation of PDL in undesired locations. Our current results (Figure 2–5) demonstrate that PDL lines act to enhance growth: processes strongly favor growth on PDL lines. Combined with our previous experiments36 and other literature reports,14,17,22 these results show that substrate-patterned proteins (PDL, laminin, L1-FC, etc.) provide a powerful tool to direct process growth and connectivity. Our experiments also demonstrate that geometrical factors influence whether neurons in close proximity to one another send out neurites and that cortical neurons can extend beyond PDL patterns onto the inhibitory PEG substrate, but only under certain conditions. First, neurons that are patterned on PDL squares within 35 $\mu m$ from one another will grow over the inhibitory PEG substrate that separates the neurons and fasciculate with processes extended from the adjoining neuron (Figure 3A). Second, neurons that are patterned on PDL squares with short (15 $\mu m$) PDL lines directed toward an adjoining square and line 100 $\mu m$ away will grow over a 70 $\mu m$ PEG gap and fasciculate with a process extending from the adjacently neuron (Figure 4B). These results suggest that there may be geometry-dependent critical separation distances, between neurons separated by a PEG-covered surface, that determine whether they will send out processes.

Figure 4. Left: schematic of patterning of PDL for samples shown. The PDL squares have sides of length 10 $\mu m$ and the PDL lines have width 2 $\mu m$. (A–D) Optical micrographs of embryonic neurons cultured for 5 days at 37°C. (A) $D = 60 \mu m$, $d = 40 \mu m$ ($L_p = 20 \mu m$); (B) $D = 100 \mu m$, $d = 70 \mu m$ ($L_p = 30 \mu m$); (C) $D = 120 \mu m$, $d = 80 \mu m$ ($L_p = 40 \mu m$); (D) $D = 170 \mu m$, $d = 90 \mu m$ ($L_p = 60 \mu m$). Other samples consistently yield the result that the neurons connect when $d \leq 70 \mu m$ and do not connect when $d \geq 80 \mu m$. Remarkably, patterning relatively short PDL lines induces neural processes to grow out even when they are separated by 70 $\mu m$ of PEG. As shown in Figure 3, when PDL lines are absent, neurons separated by more than 35 $\mu m$ fail to grow processes that connect.
To gain a better understanding of the role of geometrical constraint in neural guidance, we have systematically varied two parameters: (a) distance D between the edges of the growth squares (Figures 3 and 5, blue data points) and (b) distance d between regions with patterned proteins (PDL lines extending from regions containing neuron cell bodies) (Figures 4 and 5, red data points). Figure 5 shows plots of the length L of the longest process as a function of spacing D between neurons (blue data points) and spacing d between the edges of the protein lines (red data points).

There are several common features shown by both plots: (1) the process length decreases rapidly above a critical distance \( D_{cr} = 35 \mu m \) for no PDL lines and \( d_{cr} = 70 \mu m \) for PDL lines; (2) processes grow over the inhibitory PEG substrate under certain conditions (discussed above); (3) for separations beyond a certain length scale \( \sim 100 \mu m \) very little process growth occurs. The quasi-exponential decrease of process length L over the PEG substrate with separation for \( D > D_{cr} \) and \( d > d_{cr} \) (Figure 5A) and the fact that giving a vector of growth (PDL line) causes the process to be likely to continue to grow in that direction both suggest that growth of neural processes over the PEG is governed by diffusion processes. Many characterized as well as novel secreted substances could bind directly to receptors on adjacent neurons, stimulating them to grow preferentially toward the neuron secreting such substances.

By fitting the blue data points in Figure 5 with an exponential decaying function for \( D > D_{cr} \)

\[
L = L_0 \exp \left( -\frac{D}{x_0} \right)
\]  

we obtain

\[ L_0 = 130 \mu m \text{ and } x_0 = 18.7 \mu m \]  

Assuming a diffusion process with the length scale \( \langle \chi \rangle^{1/2} = (4D_{cr}x_0)^{1/2} \) a reasonable time scale in the range \( t_0 = 1-100 \text{ s} \), and setting \( \langle \chi \rangle^{1/2} = x_0 = 18.7 \mu m \), we obtain a diffusion coefficient: \( D_{cr} = 10^{-7}-10^{-8} \text{ cm}^2/\text{s} \). This diffusion coefficient is consistent with diffusion processes of most proteins of intermediate mass in water. We also note that the value of \( L_0 = 130 \mu m \) obtained from the data is close to the threshold distance of 100 \( \mu m \) for process outgrowth from the cell body in different geometries (see discussion above and ref 36). Similar results are obtained from the data represented by red squares (patterns with PDL lines) in Figure 5A.

Figure 5B is also consistent with a directed growth response promoted by the presence of secreted substances. The data represented by blue squares show that, in the absence of PDL lines, the number N of processes decreases with increasing separation D between cells and that above 100 \( \mu m \) separation very little process outgrowth occurs. The data represented by red squares show the same behavior occurs for patterns with PDL lines for separations d above the threshold value \( d_{cr} = 70 \mu m \), that is, for cases when the neurons do not connect. However, for patterns where neurons make at least one connection \( d < d_{cr} \) N is independent of d, suggesting the presence of additional secreted substances that bind the substrate when PDL patterns are present (discussed below). We mention that the difference in shape between the curves formed by the blue data points in Figure 5A,B indicates that different signaling molecules (different diffusion coefficient) might be present in the two cases. In other words, the signaling molecules involved in promoting the length of a process could be of different type than the molecules inducing the growth in the number of process. Indeed, many possible types of signaling molecules are involved in neuronal growth (see below). However,
while consistent with this hypothesis our data cannot be used to prove it. In contrast to Figure 5A, the data for samples with no PDL lines shown in Figure 5B do not have a well-defined turning point and therefore cannot be used to determine a diffusion coefficient.

Cortical neurons can extend lengthy (∼20 μm) fingerlike protrusions, called filopodia, that have been shown to be critical precursors for the formation of neurites.53 Thus, it is possible that long filopodia extend from adjacent neurons, without adhering to the substrate, and adhere to one another, inducing a programmed maturation of the filopodia into neurites. The process of filopodium maturation into a neurite is not well characterized, mainly because it has been difficult to follow the process over time, due to the unpredictable growth patterns of neurites on unpatterned surfaces. However, it is known that the maturation of a filopodium into a neurite must be accompanied by maturation of the cytoskeleton (actin filaments and microtubules) to allow directed transport of material (membranous vesicles, proteins, etc.) into the nascent neurite.9 Generally, filopodia extending from spherical cell bodies consist of parallel bundles of actin filaments (f-actin), and although microtubules (MTs) can invade filopodia transiently, they are not present for more than a few seconds to minutes.54 Neurites are comprised primarily of MTs and have few actin filaments. Thus, the invasion of MTs and their subsequent bundling is a marker for neuritogenesis. However, the distance of PEG over which neurites can bridge (∼70 μm) in our experiments is longer than that over which cortical neuron filopodia are generally thought to extend (20 μm each or ∼40–50 μm total). Thus, it is unknown how neurites can extend over such a long distance of inhibitory PEG. Therefore, it is possible that growth cones secrete substances that modify the PEG substrate to allow for growth, in addition to secretion of diffusible substances discussed above. One potential substance is laminin, an important component of the extracellular matrix (ECM), that neurites themselves may release during outgrowth.60,61 We note that it is unlikely that laminin alone would be sufficient to allow growth on PEG since we have shown that incubation of nanoshaved PEG with laminin only allows growth on the shaved areas. Nevertheless, growing neurites likely secrete many factors, some of which might change the PEG enough for neurally secreted laminin to adhere. Such substances might include proteoglycans or glycolipids that may themselves adhere to the PEG and allow binding of laminin.

In addition to potential secreted substances that bind the substrate there is the possibility that cortical neurons secrete diffusible substances that bind directly to receptors on adjacent neurons, stimulating them to grow preferentially toward the neuron secreting such substances. Many characterized, as well as novel, secreted substances could be involved in a directed growth response.57–59 However, as far as the authors know, there is no report in the literature quantitatively measuring the amount of neurotropic chemicals released from a growth cone or axon. Nevertheless, release of guidance cues from a micropipet positioned near the axonal growth cone of extending hippocampal or cortical neurons can induce either attraction or repulsion of the growth cone toward or away from the pipet, respectively.52,53 One potential methodology that could be applied to measure what is being released from the growth cone/distal axon is to grow neurons in a microfluidic chamber and collect the media in the growth cone chamber and then perform an ELISA assay for specific proteins or mass spectroscopy measurement for all proteins. Furthermore, numerical simulations of theoretical models of chemotactic response64–66 can incorporate systematic variations of the experimental conditions and therefore enable a more comprehensive test of their relevance and domain of applicability. These dynamical models involve the entire time evolution of the growth and require real-time imaging of the system during growth. These experiments are currently under investigation and can provide important information about the underlying processes giving rise to the ultimate morphology.

Conclusions

In this study we have used AFM lithography for controlling the location, growth, and connectivity of cortical neurons (cultured from mouse embryos) on gold surfaces. This AFM patterning method enables the simplification of the growth environment of the neurons, so that fundamental processes governing their behavior are easier to elucidate. Our results show that neuronal growth depends reproducibly on the geometry of the patterning substrates and that substrate patterning can direct process growth beyond the physical extent of the pattern itself. These experiments reveal systematic and unexpected effects of patterning geometry on process growth, providing strong evidence that the presented approach might yield new insight into the fundamental processes that govern growth and development of the central nervous system neurons.

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Supporting Information Available: Epifluorescent images taken on control samples. This material is available free of charge via the Internet at http://pubs.acs.org.

(64) Xu, J.; Rosoff, W. J.; Urbach, J. S.; Goodhill, G. J. Development 2005, 132, 4545.