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Positioning and guidance of neurons on gold surfaces by directed assembly of proteins using Atomic Force Microscopy

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ABSTRACT

We demonstrate that Atomic Force Microscopy nanolithography can be used to control effectively the adhesion, growth and interconnectivity of cortical neurons on Au surfaces. We demonstrate immobilization of neurons at *well-defined* locations on Au surfaces using two different types of patterned proteins: 1) poly-D-lysine (PDL), a positively charged polypeptide used extensively in tissue culture and 2) laminin, a component of the extracellular matrix. Our results show that both PDL and laminin patterns can be used to confine neuronal cells and to control their growth and interconnectivity on Au surfaces, a significant step towards the engineering of artificial neuronal assemblies with well-controlled neuron position and connections.

1. Introduction

In the developing brain extracellular matrix proteins and guidance factors provide critical signals for controlling the path that growing neurites follow to reach their targets. Neuronal cells develop two types of neurites (processes): long, threadlike axons that carry information to target cells and shorter, thicker, dendrites that receive inputs from other neurons. Extensive research using traditional biological techniques has provided valuable information regarding the neuronal response to individual biochemical and topographical guidance cues. For example it is known that *in vivo*, axonal navigation is not just an intrinsic "program of directions" but it also relies on external molecular cues (extracellular proteins, guidance factors) whose precise spatial arrangement plays a crucial role in guiding neurites to their targets [1–4]. Moreover, it has been discovered that disruption of neuronal adhesion can cause many diseases, including birth defects, mental disorders, and sensory and motor deficits [1–4]. However, the local environment faced by the growing neuron is inherently rich and contains a complex array of guidance cues whose collective influence on the growing neuron is still poorly understood. In addition, the extremely complex neuronal architecture found *in vivo*, with millions of neurons making thousands of unique connections makes a detailed study of the specificity of synaptic connections a daunting, if not impossible, task.

Therefore, an alternative approach is often used that focuses on developing methods for reproducing such molecular patterns *in vitro*. This approach aims at creating simpler, artificial neuron networks by reducing the number of neurons in the sample, and by controlling both the location of neuron somata and the direction of process growth. There are several major challenges that these fabrication methods have to overcome to reproduce successfully the molecular patterns encountered by neurons *in vivo*: a) the low concentration of biomolecular cues involved, b) their precise spatial arrangement, and c) the maintenance of biological function and stability of biomolecules over the relatively long periods of time (days) involved in neuronal growth. Moreover, since neurons are known to alter their behavior dramatically in response to even slight changes in their environmental surroundings [5–7] one must also be able to regulate tightly the external conditions such as temperature and gas content.

To date, the control and study of neuronal development *in vitro* has been achieved either by plating neurons on a uniform substrate of adhesion molecules, such as laminin or fibronectin, or on

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a micropatterned substrate of these molecules created by photolithography, photoresist protection, or micro-contact printing [8–20]. Although these experiments can give valuable information about general growth conditions and the interactions between neurites and various growth substrates [9,11,12,14], they are very far from reproducing the complex molecular environment faced by neurons *in vivo*. In the absence of *molecular scale* chemical cues neurites tend to grow in random directions, making controlled investigation of synapse formation and synaptic interactions between neuronal cells extremely difficult. Micropatterning techniques offer (in principle) the ability to define and manipulate the environment of single cells [6,13–20]. For example, micro-contact printing was used to produce discontinuous gradients of substrate-bound chemorepulsive cues (ephrinA5) [14], and to selectively pattern the extracellular domain of the cell adhesion protein L1 on poly-lysine coated glass substrates [13]. Microfabrication has also been used to investigate the role of substrate geometry [6,15,16,19,20], and to achieve dynamic control of the local temperature of the neuronal cells [5]. At present, however, a major drawback for most of these neuronal micropatterning techniques is that, having their origin in silicon technologies used in microchip industry, they require processes that are likely to alter the biochemical activity of the patterned proteins, such as drying steps, exposure to organic solvents, and thermal treatment, to name a few. Moreover, low density neuronal cultures (necessary for studying individual cell–cell signaling) are extremely hard to maintain on these micropatterned devices [21].

This paper introduces a new approach for controlling the adhesion, growth and interconnectivity of cortical neurons on Au surfaces. Specifically, we use Atomic Force Microscopy (AFM) nanolithography [22–26] to immobilize two different types of proteins at *well-defined* locations on Au surfaces: 1) poly-D-lysine (PDL), a positively charged polypeptide used extensively in tissue culture and 2) laminin, a component of the extracellular matrix. Previous reports have shown that both types of proteins promote the adhesion of neuronal cells on surfaces, with laminin also acting as a promoter for neurite growth [8,12,16,17,27]. Our results show that both PDL and laminin patterns can be used to confine neuronal cells and to control their growth and interconnectivity on Au surfaces. We also show that AFM nanolithography provides unique advantages for this type of work: 1) a high degree of control over location and shape of the protein patterns is achieved, 2) the procedure is carried out in aqueous solutions (protein buffers), such that the proteins are very likely to retain their folding conformation/bioactivity, and 3) minimum protein feature size can be reduced down to several tens of nm (typically ~50 nm) [25,26].

2. Experimental methods

2.1. Overview

Long chain poly (ethylene) glycol polymers self-assembled on Au are known to be very efficient in resisting both protein and cell adhesion [27–29]. For the experiments reported in this paper we have used undecanethiol triethylene glycol: HSC_{11}-EG_3 (referred to below as PEG) as a protein-resistant and cell-resistant SAM [25,26]. To control the locations where neurons adhere to the Au surface we use an AFM-based nanolithography technique called nanoshaving [24,30] (Fig. 1). Briefly, in nanoshaving, previously selected regions of the PEG SAM are removed ("shaved") from the Au surface by the AFM tip, while the tip is scanning these regions at high force (~100 nN). This step is done in a pure solvent solution (typically ethanol). The sample is then removed from the solvent and immersed into a buffer solution containing the proteins of interest (either laminin or PDL for the experiments reported in this paper). Since the PEG resists protein adhesion, the proteins will attach to the Au substrate only onto those areas where the PEG was removed by nanoshaving (Fig. 1B). Thus this nanofabrication method allows one to create patterns of surface-bound proteins, with various shapes and sizes (from ~10 μm down to ~50 nm), and positioned at precise locations onto the Au substrate.

2.2. Substrate and SAM preparation

The Au surfaces were prepared by e-beam evaporation on mica substrates in a vacuum chamber (Denton Vacuum, model DV 502-A) at a background pressure below 2×10^{-6} Torr. For most samples the mica (Clear ruby Muscovite, mica New York Corp.) was preheated to 350 °C before deposition by using two quartz lamps which are mounted behind the mica. After evaporation, Au films were annealed at 360 °C under vacuum for 30 min and then cooled to room temperature. After removal from chamber, the Au-coated mica substrates were annealed in a H_2 flame for 2 min to coalesce the gold grains. For comparison (see Section 3.4), we have also prepared several samples by evaporating Au/mica at room temperature without any prior annealing. For all samples 50 nm of gold (Alfa Aesar, 99.999% purity) was deposited on freshly cleaved mica at the rate of 2–3 Å/s. After metallization, the Au-coated mica was immersed into 0.1 mM undecanethiol triethylene glycol (PEG) (Prochimia, 98% purity) solution for 48 h, so that a compact monolayer was allowed to form on the Au (111) surface. Before it was characterized by AFM, the sample was rinsed for 2 min with pure ethanol (AAPER Alcohol and Chemical Co.) and dried by a mild flow of nitrogen.

2.3. AFM lithography

AFM lithography and imaging were carried out using a Digital Instruments MultiMode AFM (Santa Barbara, CA) with a Nanoscope IIIa controller and Type J scanner (Digital Instruments). All AFM experiments were performed in contact mode, in a liquid cell kept at room temperature using commercially available V-shaped cantilevers with oxide-sharpened Si_3N_4 tips (NPS, Veeco Instruments, 0.58 N/m spring constant). To create PDL regions on PEG/Au substrates, we use a three-step AFM-based technique called nanoshaving (Fig. 1) [24,30]. In the first step an AFM tip is used, at low force, to image the surface topography and select a certain region on the PEG/Au substrate. Second, the PEG within a micron-size region is shaved away by the AFM tip under relatively high force loads (Fig. 1A), while the SAM is in contact with a pure solvent (ethanol). The displaced PEG molecules ejected into the solution become extremely diluted and have little opportunity of returning to the gold surface. Finally, the ethanol solution is replaced with a buffer solution containing PDL (1 mg/mL poly-D-lysine 34 kD, Sigma in 0.1 M sodium borate, pH 8.5), or laminin (0.71 mg/mL laminin, 850 kDa, Invitrogen in 50 mM Tris–HCL + 0.15 M NaCl, pH 7.4) and the sample is incubated in this solution for 45 min (Fig. 1B). Since the PEG is very efficient in resisting protein adsorption [25,28,29], the proteins can adhere to the Au substrate only on the regions where this SAM was removed by nanoshaving (Figs. 1B and 2).

2.4. Cell culture, immunocytochemistry and imaging

All the experiments reported in this paper use cortical (E14.5–16.5) neurons obtained from Swiss Webster mice [32,33]. All mouse procedures were approved by the University of Wisconsin Committee on Animal Care and were in accordance with NIH guidelines. Briefly, the cells were dissociated by treatment with trypsin (25%, 15 min, 37 °C), then triturated with a micropipette tip, diluted in plating medium (Neurobasal medium with 5% FBS Hyclone, B27 supplement, 2 mM glutamine, 37.5 mM NaCl and 0.3% glucose) [32] and plated at low density (2000 cells/cm^2) onto the PDL/PEG/Au substrates. After 2 h the samples were flooded with Serum Free Medium (plating medium without FBS) and incubated for 96–120 h. All cultures were fixed in 4% paraformaldehyde/Krebs/Sucrose Fixative [32,33] at pH 7.4 for 15 min, then rinsed three times with phosphate buffered saline (PBS) solution. Optical-microscope images were collected using a Nikon Eclipse ME 600 microscope.

3. Results

3.1. Patterning proteins into PEG layers by AFM lithography

Fig. 2A shows an AFM image of a typical PDL pattern made by nanoshaving. Here the PDL proteins self-assemble in a patterned 10 × 10 μm square, surrounded by the PEG SAM. The surface roughness of these PDL patches (measured from the AFM height images) is comparable to the roughness measured on PDL layers self-assembled on bare Au surfaces. This result suggests that the mechanism of PDL self-assembly is the same on both AFM patterned patches and bare Au surfaces.

Protein patterns such as the one shown in Fig. 2A (referred to as "growth squares" throughout the paper) are used to control the position of neuronal cell bodies on the Au substrate (see Section 3.2 below). Fig. 2B shows an AFM image of a different type of pattern: a growth square (bottom) similar to the one shown in Fig. 2A, with a PDL "growth line" extending outwards. This type of pattern is

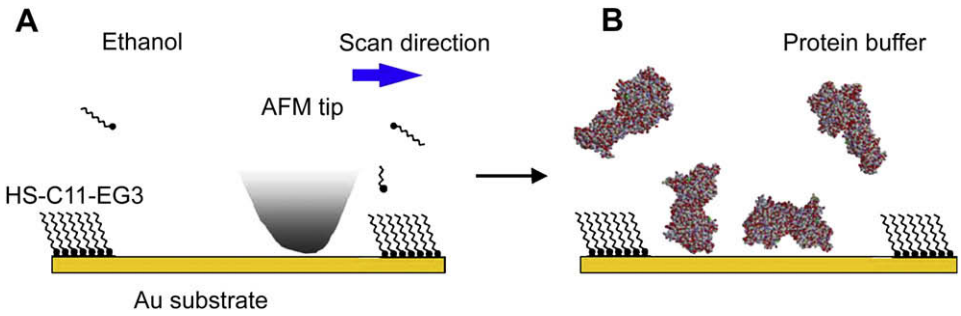

Fig. 1. Nanoshaving (schematic). (A) The AFM tip is scanning a selected PEG/Au region at high force, effectively removing (shaving) the PEG SAM from this area. (B) The substrate is then immersed in a buffer solution containing the proteins (laminin or PDL), which adhere (physisorption) to the Au surface only onto the PEG-free (nanoshaved) region. The proteins schematic shows lysine monomers generated using protein data bank [31].

used for axonal guidance (see Section 3.2). As in [6], we refer to the surface patterns that restrict the physical area on which neurons can attach and grow as "islands" and "bridges." Cell bodies adhere to the larger (>3 μm) islands since neurons need a minimum surface area to attach and spread out. A bridge pattern will only allow processes to grow on it, while blocking attachment and migration of cell bodies. This concept is often called "size exclusion principle" [6].

3.2. Control of neuronal growth and interconnectivity on protein/PEG patterned Au substrates

Previous reports [27] and our control experiments (data not shown) demonstrate that neurons do not adhere to PEG polymers self-assembled on Au. However, many studies have shown that both positively charged poly-amino acids, such as PDL, and extracellular matrix proteins, such as laminin, promote cell adhesion on various surfaces [8,12,16,17,27]. We have therefore used AFM patterned PDL and laminin patches (see Figs. 2 and 3) to control the location of neuronal cells on PEG-coated Au substrates.

Fig. 3 shows neuronal cells grown on 5 PDL growth squares (all similar to the growth square shown in Fig. 2A) arranged in an "L-shaped" pattern. The growth squares were patterned by nanoshaving 100 μm apart into the surrounding PEG/Au substrate. The image shows 6 cell bodies (labeled 1–6), that are *exclusively* confined to the PDL patterns with no cells adhering to the surrounding PEG, demonstrating that neurons can be patterned at precise locations on PEG/Au substrate. In addition, there are no processes growing out from the cell bodies, showing that isolated neurons on non-adhesive PEG/Au substrates do not form neurites.

Previous work has also demonstrated that neuronal cell bodies require a minimum surface area (>2 μm) to form stable adhesions with the substrate [16–18]. We show that these results can be used to guide neurite growth on PEG/Au surfaces. More precisely, we use nanoshaving to pattern protein (PDL or laminin) lines extending outward from the growth squares (Fig. 2B). Typically, each protein line is 40–45 μm long and 2 μm wide; that is, they are narrow enough to minimize the initial adhesion of cell bodies when plated [16–18], yet wide enough to act as neurite paths for process outgrowth [6]. Fig. 4A shows an example of two neurons grown on a pair of PDL line/square patterns. As expected, the two cell bodies (marked 1 and 2 in Fig. 4A) attach to the PDL squares, while each cell extends a single dominant process (as is typical of axons [12,18]) along the PDL lines (schematically shown by blue lines in Fig. 4A. In addition, there are several shorter processes growing around the surface of the cells. A similar result for laminin is shown in Fig. 4B. These results show that axonal growth is guided by adhesive protein (PDL or laminin) growth lines. Once its formation

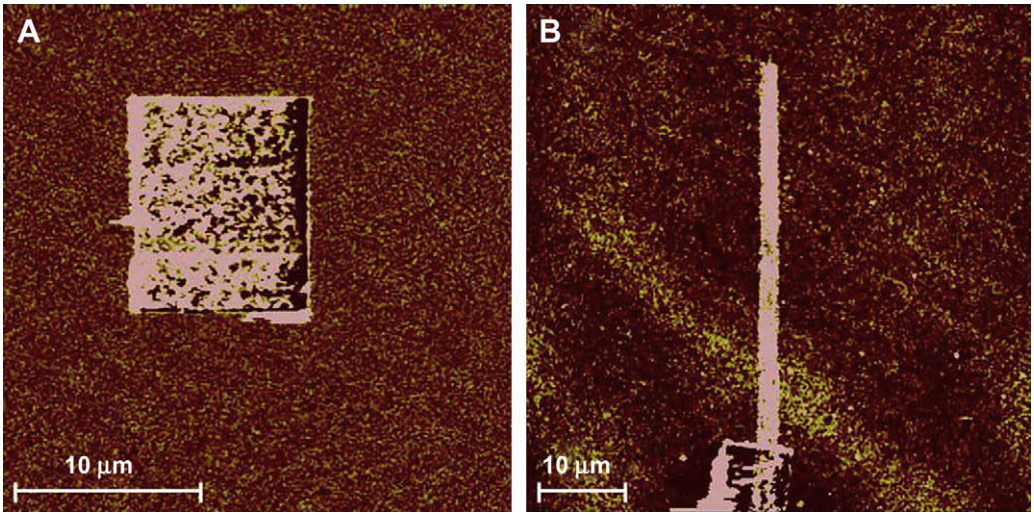

Fig. 2. AFM images of typical protein patterns made by AFM lithography (nanoshaving). (A) AFM height image of a 10 × 10 μm poly-D-lysine (PDL) square made by nanoshaving. The protein pattern is surrounded by undecanethiol triethylene glycol (PEG) self-assembled monolayer (SAM). PDL promotes cell adhesion while the PEG forms a cell-resistant SAM. Therefore, these patterns can be used to control the location of neuronal cell bodies on the substrate. (B) AFM height image of a 2 μm × 45 μm PDL square/line made by AFM lithography. These patterns are used for guiding neurites (see text).

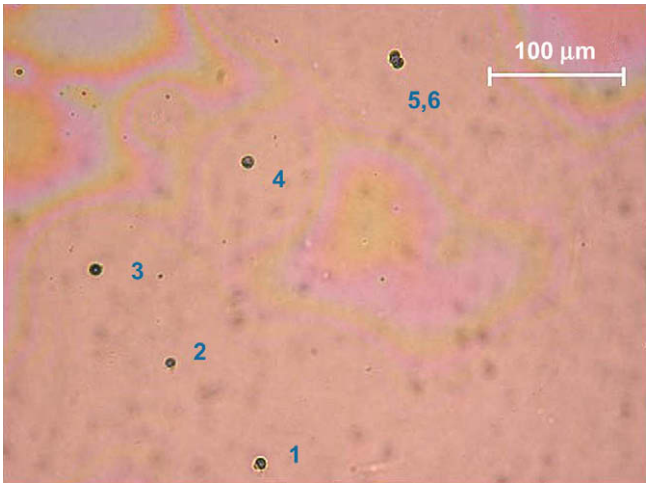

Fig. 3. Optical micrograph of neuronal cells immobilized in an "L-shaped" pattern made from 5 PDL growth squares similar to the one shown on Fig. 2A, demonstrating the high degree of control of neuronal cell growth obtained using nanoshaving patterning. The cells were incubated for 5 days before fixation in paraformaldehyde solution. The numbers and the scale bar were added to the optical image.

is initiated the axon continues to grow in the direction of the protein pattern, until it eventually arrives in the proximity of another cell (Figs. 4 and 5A).

3.3. Large scale patterning of neuronal growth

We demonstrate the utility of this AFM-based lithography method by using it to create large scale neuron networks on PEG/Au surfaces. We show that nanoshaving allows a high degree of control over both the locations of neuronal cell bodies and over the direction of neurite growth. For example, Fig. 6 shows PDL (Fig. 6A) and laminin (Fig. 6B) growth squares, arranged in an L-shape, and patterned on a wide area (~500 μm × 500 μm). The growth squares are spaced 100 μm apart, and for each pair of adjacent squares we patterned a pair of protein growth lines (line dimensions: 45 μm length, 2 μm width) oriented along the direction that connects the two squares (similar to Fig. 4).

For Fig. 6A 8 PDL squares and 14 PDL lines were patterned via nanoshaving. We found a total number of 12 neurons (labeled 1–10 and a,b) attached to the substrate. Neurons labeled 1–10 (83% of the total) adhere to the substrate on the patterned PDL squares (neuron pair 3,4 adhere at the same site, as is also the case for the pair 6,7). We estimate that the percentage of post-mitotic neurons at this stage is 80–90% [33]. Therefore the neuron pairs observed at a given PDL site (pair 3,4 or 6,7), are formed either from one pre-mitotic neuron which adheres at this site and then divides or from two post-mitotic neurons that adhered at the same time. In the current experiments we cannot differentiate between these two possibilities. All cells that adhered to a somal attachment site (PDL square) develop a process longer than 40 μm that is oriented in the direction of the of the patterned PDL growth line. Typically, the neurons adhering to the PDL squares also exhibit two or more minor processes < 20 μm in length (that normally are precursors of dendrites). Finally, 17% of the neurons (a,b) adhere to the substrate outside the patterned areas. We hypothesize that these are sites where PDL adheres to Au due to existing defects in the underlying PEG/SAM. Indeed, we show below that defects can be created artificially by AFM scanning or by using unannealed Au substrates (see Section 3.4).

Fig. 6B shows neuronal growth on a similar surface, with 8 laminin growth squares and 14 growth lines patterned via nanoshaving. An analysis similar to the one presented for Fig. 6A shows that a total number of 7 cells attach to the substrate, 100% adhering on the patterned squares (there is no neuron adhering to the site #3). 85% of neurons develop at least one long process (>50um μm) oriented in the direction of a patterned laminin growth line. These long processes form a surface network connecting most pairs of cells (with the exception of the pair labeled 5,6). We also note that neurons attached to laminin growth squares develop a much smaller number of minor processes than the cells grown on PDL patterns (Fig. 6A).

An AFM survey conducted on the samples after fixation of the neurons shows that between 10 and 30% of the area of the underlying protein squares is left uncovered by the cell body (Fig. 5B). This "edge effect" is typically found around cells grown on PDL squares that also develop several minor processes (40% of the cases). We have found that the edge effect is less common (20% of cases) for cells grown on laminin, which also tend to have asymmetric cell bodies and to develop fewer secondary processes.

3.4. Influence of the AFM imaging and the quality of Au substrate on the resulting neuron patterns

We have found that increasing the number of AFM scans (in contact mode) for imaging a certain area of the PEG/Au substrate leads to an increase in the number of defects (random sites where

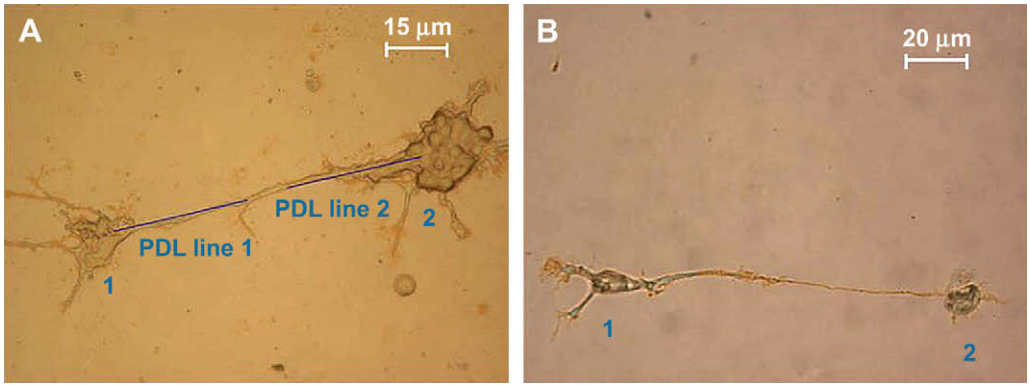

Fig. 4. (A) Optical micrograph of two cortical neurons immobilized on PDL growth squares and connected by two long processes (as is typical of axons). The axonal growth is promoted by PDL growth lines (see Fig. 2). The two solid lines (schematic) show the direction/length of the underlying PDL growth lines. (B) A similar result as the one presented in Fig. 4A, obtained for neurons grown on two laminin growth squares/lines. The underlying laminin lines are patterned in the direction connecting the two neurons (here for clarity, the schematic of these lines is not shown as in Fig. 4A).

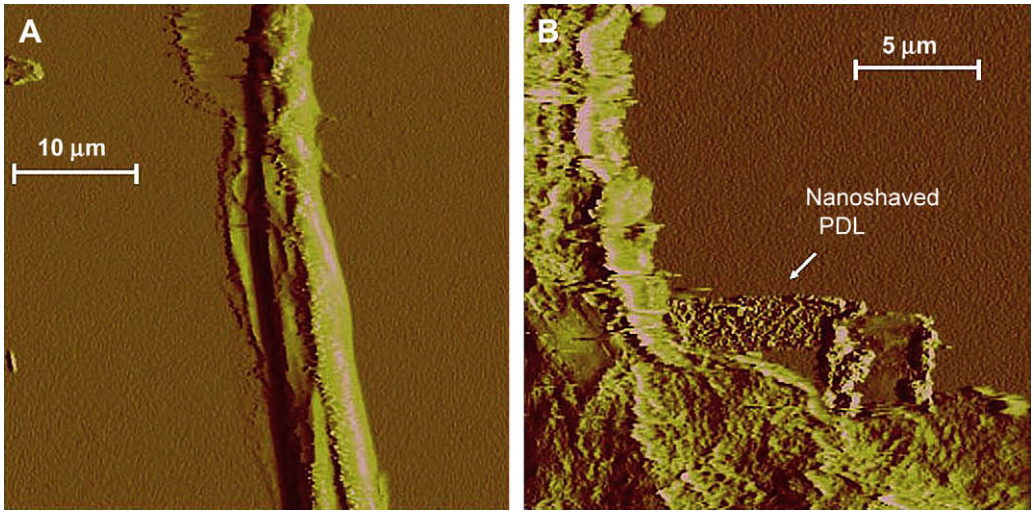

Fig. 5. (A) AFM deflection image of a long process (axon) whose growth is controlled by a patterned PDL line patterned on a sample similar to the one shown in Fig. 7A. The axon continues to grow in the direction of the patterned protein line. The underlying PDL pattern is 2 μm wide and 45 μm long. (B) AFM deflection image of a neurite growing out of the cell body. PDL at the edge of the growth square that is not covered by the cell body/neurite is visible in the AFM images. Uncovered protein at square edges is visible for 40% of the neurons grown on PDL and 20% of the neurons grown on laminin. We hypothesize that the presence of uncovered protein is related to the growth of secondary processes (see discussion).

the PEG is removed, exposing the bare Au surface) in the PEG, even when the imaging is done at low forces (5–10 nN). These AFM-generated defects become sites where proteins adhere to the Au substrate, therefore leading to an increase in the number of cells adhering to the substrate at random locations. For example, Fig. 7A shows a 100 × 100 μm area with 5 PDL patterned squares. The whole area was imaged 10 times (with the sample in ethanol) prior to immersing the sample in PDL buffer.

Fig. 7B shows the resulting neuron growth on the area shown in Fig. 7A. In addition to the 5 cells that adhere to the patterned growth squares (L-shape), many neurites (and possibly some dissociated cell bodies) grow (adhere) randomly inside the AFM-imaged area. This result is in contrast to the neuron patterns shown in Figs. 3,4 and 6, where the number of actual scans was minimized (1 scan/pattern) resulting in very few cells (fewer neurites) adhering (growing) outside the patterned squares (lines). For the patterns shown in Figs. 3,4 and 6, the 10 × 10 μm area defining each PDL square was scanned only once at high force (100 nN), necessary to remove the PEG, then the AFM tip was retracted and moved manually to the location selected for patterning the next square.

The formation of the neuron network is also influenced by the quality of the underlying Au substrate. In particular, we found that the process of annealing the mica substrate during the Au evaporation (see Section 2.2) has a dramatic effect on the resulting neuron pattern. Annealing the substrate resulted in a 10-fold increase of the average surface area of Au grains and a greatly reduced number of hole defects (locations where the PEG SAM is disrupted or does not form on Au). This improvement of the Au film leads to better PEG coverage and to fewer possible random sites where proteins can adhere to the substrate, and therefore to many fewer neurons adhering outside the patterned areas.

4. Discussion

We have introduced an AFM-based method for patterning neurons on PEG/Au substrates that allows a high degree of control over neuron cell density, location on the substrate, and direction of neurite growth. We have shown that this method can restrict neuron cell bodies to precise locations on the substrate (Figs. 3,4 and 6). These locations are patterned (10 × 10 μm) protein squares (Fig. 2A),

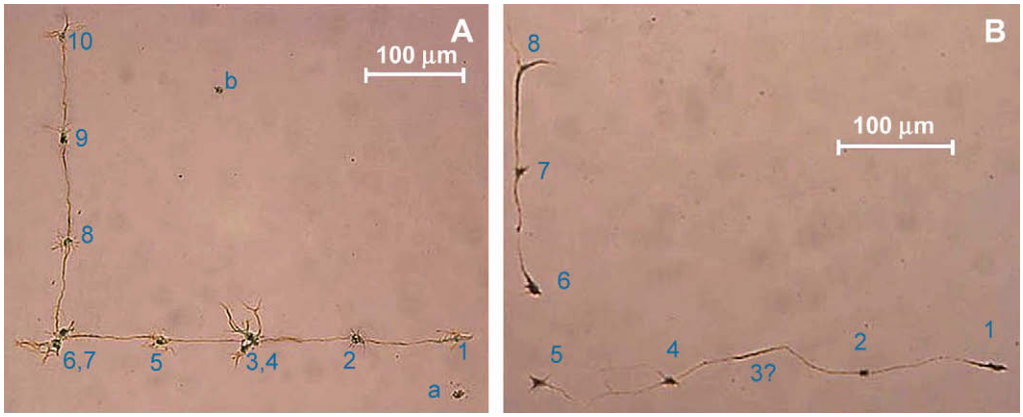

Fig. 6. Optical micrographs of large scale neuronal patterns created via nanoshaving. (A) Neuronal cells are immobilized at precise locations on patterned PDL growth squares; the growth directions of the long processes are controlled by nanoshaved PDL lines. (B) A similar result obtained for neurons grown on patterned laminin squares/lines. The neuron labeling is explained in the text.

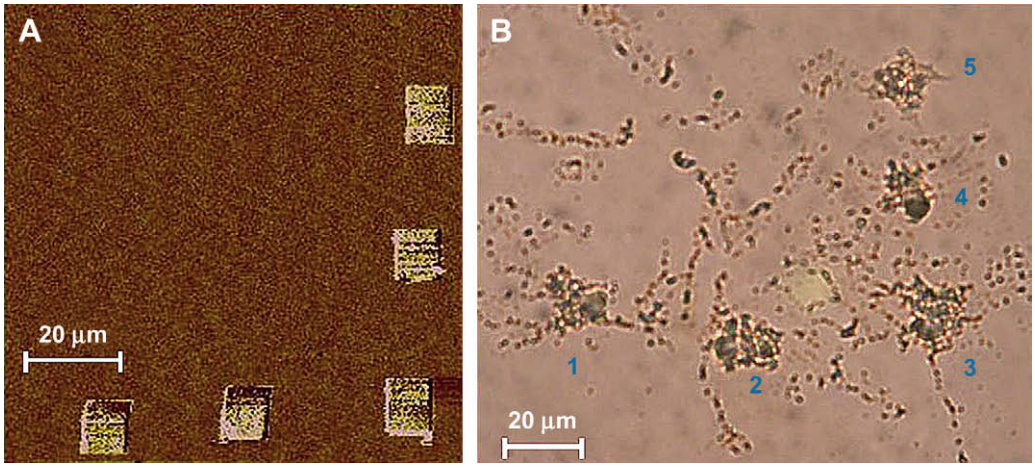

Fig. 7. AFM height (A) and optical (B) images of a sample showing the influence of increasing the number of AFM scans on the neuronal growth. This sample was AFM scanned 10 times (contact mode) before being exposed to PDL. Comparing with Figs. 3,4 and 6, which were scanned once before exposure to PDL, one sees that the number of defects induced in the PEG SAM is increasing with the number of scans therefore leading to less control over neuronal locations and growth. The 5 underlying protein squares shown in 7A are located in the proximity of the center of the remnant cells labeled 1–5.

that promote cell adhesion, and which are surrounded by a SAM of non-permissive PEG (undecanethiol triethylene glycol). Furthermore, we show that the surface immobilized cells grow long processes (as is typical of axons [12,18]) only along AFM-patterned protein lines (30–45 μm long, 1–2 μm wide) (Fig. 2B). These narrow protein lines are patterned between pairs of growth squares, and typically they cover 60–90% of the total distance between two adjacent squares (Figs. 4 and 6). By cutting across the non-permissive PEG these protein lines provide permissive "neurite paths", for the outgrowth of long processes and allow a high degree of control over both the location and growth direction of these long neurites. In addition, the patterned protein lines are narrow enough such that no cell body was observed to adhere on (or to migrate along) these lines.

Experiments reported by other groups [11,12,18] have shown that cultured neurons extend filopodia and lamellipodia around their circumference prior to growing neurites. Furthermore, filopodia at the leading edge of a growth cone adhere preferentially to certain substrates and, when they do so, they initiate the outgrowth of several neurites, which typically develop into a single long axon and multiple short dendrites [11,12,18]. A recent report also indicates that filopodia are key factors for the formation of neurites from cortical neurons [33]. Our results (Figs. 4–6) demonstrate that, once their growth is initiated, and in the absence of additional protein patterns, these long processes grow with relatively uniform diameter and extend in straight lines with no branching (Fig. 5A shows a typical AFM image of one of these processes). These findings are also in agreement with literature reports that neurons grow in straight (unbranched) processes along uniformly covered poly-L-lysine (PLL) patterns (defined by photolithography on resist coated glass coverslips), and that the axonal branching is stimulated by additional geometrical or chemical cues, such as increased contact area between the growth cone and PLL pattern or several PLL lines intersecting at a common point [13,16].

It is well known that various molecular species have dramatically different effects on the growth cone motility. For example, it has been shown that microstamped gradients of ephrinA5 can effectively stop the elongation of growth cones [14], while the extracellular domain of the adhesion molecule L1 (micropatterned as an L1-Fc chimera) can be used to selectively grow axons, whereas somata and dendrites were unresponsive to these patterns [13]. Other studies have shown that even relatively similar extracellular matrix proteins (such as laminin and fibronectin) could cause very different growth cone behavior. For example, laminin on model guideposts (polystyrene beads) produced a substantial increase in growth cone velocity, while fibronectin led to a sustained velocity decrease [8]. In this paper we have used nanoshaving to create surface patterns of both non-permissive/non-adhesive (PEG) and adhesive/permissive (PDL, laminin) molecules. In both cases we have found that isolated cortical neurons (grown on protein squares $\geq$ 100 μm apart and surrounded by PEG) do not form *any* neurites unless there are protein lines patterned between the growth squares. We have also found that, although both laminin and PDL promote cell adhesion and neurite outgrowth for cortical neurons, the morphology of the resulting cells is different (compare Fig. 4A with Figs. 4B and 6A with Fig. 6B). Neurons grown on PDL tend to have round cell bodies and multiple secondary processes grown around their circumference (Figs. 4A and 6A). In contrast, the cells grown on laminin tend to have asymmetric cell bodies and to grow fewer secondary processes (Figs. 4B and 6B). A possible explanation for these observations is that laminin, as a component of the extracellular matrix, allows more mobility for the soma as opposed to the positively charged PDL. Indeed, real time observations of neuronal growth reported in literature [11,12] have shown that, when grown on laminin, both the cell nucleus and cytoplasm rotate so as to align the cell axis (defined by a line connecting the nucleus with the main mass of cytoplasm) with the growth direction of the long process. Also, as secondary processes are grown, material (cytoplasm) is taken from the cell edges and used for the elongation of the neurite. This could involve motion/rearrangement of the cell body such that some of the corners of the underlying protein adhesion square are left uncovered, in agreement with our observations on PDL grown neurons. The dynamic of neuron growth motion/rearrangement of cell body and cell mechanics could be further studied via AFM.

Patterning extracellular matrix proteins and growth factors via AFM lithography offers several clear advantages for creating neuron networks on surfaces: 1) different types of proteins (growth factors, adhesion molecules, chemorepulsive cues etc.) can be immobilized sequentially at well-defined locations on the same substrate; 2) the molecular orientation of these proteins can be controlled [25]; 3) the procedure is carried out in aqueous solutions (protein buffers), such that the proteins retain their folding conformation and therefore their bioactivity; 4) the technique enables the investigation of regions with well-defined boundaries that are controlled at the nanoscale [25,26].

We note that the nanoshaving procedure used here for patterning protein SAMS is different from the nanografting technique for patterning submicron-scale areas by tip-induced chemical exchange with the PEG substrate [25,26] that we have reported previously [22,23]. For the experiments presented in this paper, PDL (or laminin) is *physisorbed* onto Au on a *large scale* (~ 10 μm). This is contrasted to the reported 100–500 nm patterns made by nanografting, in which cysteine-modified proteins are directly attached to Au via *covalent* (S–Au) bonds [25,26,34,35]. Given both the relatively large area of the nanoshaved PDL (laminin) patterns and their non-covalent attachment to Au, we expect these proteins to self-assemble via a very different mechanism than the one reported in nanografting experiments [22,23]. Indeed, the AFM images of the PDL patches (Fig. 2) show cluster formation, suggesting that these proteins diffuse on the Au surface before reaching their equilibrium configuration. The time scale for pattern formation (45 min–1 h) is also consistent with diffusion-controlled self-assembly. In contrast, our previous experiments [25,26] show that proteins covalently attached to Au via nanografting form very uniform patches on much shorter (~ seconds) time scales. A detailed investigation of both mechanisms will be presented elsewhere.

The ability to control both neuronal location and growth with high spatial resolution enables a variety of applications in neuroscience. First, we anticipate that it should prove to be relatively easy to use AFM lithography to immobilize other extracellular matrix proteins (fibronectin, L1-FC, ephrin etc.) on similar substrates. Furthermore, these proteins can be immobilized inside pre-patterned microfluidic channels, and therefore can be used to create complex arrays of chemical and geometrical patterns, which form guidance cues and decision matrices (locations where changes in local chemical and geometrical environment modulates axonal branch formation) on *the same substrate*. This in turn will greatly facilitate the study of synaptogenesis, since the growth of individual processes can be directed and controlled with high spatial resolution. Second, protein patterns can be created such that neurons adhere and grow processes in the vicinity or over electrodes on a pre-patterned electrode array, therefore enabling a high degree of control in studying the electrophysiological activity of individual pairs of neurons. Thirdly, local geometric and chemical control of patterned adhesion molecules has been shown to modulate cell adhesion, in the case of endothelial cells [36]. Similarly, it is hypothesized that individual neurons attach to the adhesion proteins only at discrete points on the cell body [6]. Therefore reducing the protein island size via nanoshaving could provide essential information about the location and the role played by these adhesion points. In addition, the ability to reduce the minimum size of the protein patterns opens up the possibility of fabricating steeper protein gradients, thus enabling a complete study of how the growth cones can distinguish different slopes and of the characteristic length scales for discriminating between different protein concentrations.

5. Conclusion

We have presented an AFM-based method for patterning proteins to designed micro and nanogeometry. Proteins in those microstructures remain active, and micropatterned substrates can independently control cell locations and neurite growth. We found that on both PDL and laminin nanofabricated substrates the cell bodies are almost exclusively confined on the patterned protein squares, while the growth of the long processes is highly restricted along the directions defined by patterned protein lines. The methods introduced in this work may have significant impact for engineering neuron networks and neuron based devices.

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Appendix

Figures with essential colour discrimination. All of the figures in this article may be difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.03.027.

References

[1] Sanes DH, Reh TA, Harris WA. Development of the nervous system. Burlington, MA: Elsevier Academic Press; 2006.
[2] Huber AB, Kolodkin AL, Ginty DD, Cloutier JF. Signaling at the growth cone: ligand–receptor complexes and the control of axon growth and guidance. Annu Rev Neurosci 2003;26:509–63.
[3] Charron F, Tessier-Lavigne M. Novel brain wiring functions for classical morphogens: a role as graded positional cues in axon guidance. Development 2005;132:2251–62.
[4] Kamiguchi H. The role of cell adhesion molecules in axon growth and guidance. Adv Exp Med Biol 2007;621:95–103.
[5] Pearce TM, Wilson JA, Oakes SG, Chiu SY, Williams JC. Integrated microelectrode array and microfluidics for temperature clamp of sensory neurons in culture. Lab Chip 2005;5:97–101.
[6] Pearce TM, Williams JC. Microtechnology: meet neurobiology. Lab Chip 2007;7:30–40.
[7] Potter SM, DeMarse TB. A new approach to neural cell culture for long-term studies. J Neurosci Methods 2001;110:17–24.
[8] Kuhn TB, Schmidt MF, Kater SB. Laminin and fibronectin guideposts signal sustained but opposite effects to passing growth cones. Neuron 1995;14:275–85.
[9] Kalil K, Dent EW. Touch and go: guidance cues signal to the growth cone cytoskeleton. Curr Opin Neurobiol 2005;15:521–6.
[10] Dent EW, Barnes AM, Tang F, Kalil K. Netrin-1 and semaphorin 3A promote or inhibit cortical axon branching, respectively, by reorganization of the cytoskeleton. J Neurosci 2004;24:3002–12.
[11] Smith CL. The initiation of neurite outgrowth by sympathetic neurons grown in vitro does not depend on assembly of microtubules. J Cell Biol 1994;127:1407–18.
[12] Smith CL. Cytoskeletal movements and substrate interactions during initiation of neurite outgrowth by sympathetic neurons in vitro. J Neurosci 1994;14:384–98.
[13] Oliva Jr AA, James CD, Kingman CE, Craighead HG, Banker GA. Patterning axonal guidance molecules using a novel strategy for microcontact printing. Neurochem Res 2003;28:1639–48.
[14] von Philipsborn AC, Lang S, Loeschinger J, Bernard A, David C, Lehnert D, et al. Growth cone navigation in substrate-bound ephrin gradients. Development 2006;133:2487–95.
[15] Johansson F, Carlberg P, Danielsen N, Montelius L, Kanje M. Axonal outgrowth on nano-imprinted patterns. Biomaterials 2006;27:1251–8.
[16] Withers GS, James CD, Kingman CE, Craighead HG, Banker GA. Effects of substrate geometry on growth cone behavior and axon branching. J Neurobiol 2006;15(66):1183–94.
[17] Corey JM, Wheeler BC, Brewer GJ. Compliance of hippocampal neurons to patterned substrate networks. J Neurosci Res 1991;30:300–7.
[18] Stenger DA, Hickman JJ, Bateman KE, Ravenscroft MS, Ma W, Pancrazio JJ, et al. Microlithographic determination of axonal/dendritic polarity in cultured hippocampal neurons. J Neurosci Methods 1998;82:167–73.
[19] Francisco H, Yellen BB, Halverson DS, Friedman G, Gallo G. Regulation of axon guidance and extension by three-dimensional constraints. Biomaterials 2007;28:3398–407.
[20] Dowell-Mesfin NM, Abdul-Karim MA, Turner AM, Schanz S, Craighead HG, Roysam B, et al. Topographically modified surfaces affect orientation and growth of hippocampal neurons. J Neural Eng 2004;1:78–90.
[21] Millet LJ, Stewart ME, Sweedler JV, Nuzzo RG, Gillette MU. Microfluidic devices for culturing primary mammalian neurons at low densities. Lab Chip 2007;7:987–94.
[22] Liu GY, Xu S, Qian Y. Nanofabrication of self-assembled monolayers using scanning probe lithography. Acc Chem Res 2000;33:457–66.

[23] Liu M, Amro NA, Liu GY. Nanografting for surface physical chemistry. Annu Rev Phys Chem 2008;59:367–86.
[24] Xu S, Liu GY. Nanometer-scale fabrication by simultaneous nanoshaving and molecular self-assembly. Langmuir 1997;13:127–9.
[25] Staii C, Wood DW, Scoles G. Verification of biochemical activity for proteins nanografted on gold surfaces. J Am Chem Soc 2008;130:640–6.
[26] Staii C, Wood DW, Scoles G. Ligand-induced structural changes in maltose binding proteins measured by atomic force microscopy. Nano Lett 2008;8:2503–9.
[27] Branch DW, Wheeler BC, Brewer GJ, Leckband DE. Long-term stability of grafted polyethylene glycol surfaces for use with microstamped substrates in neuronal cell culture. Biomaterials 2001;22:1035–47.
[28] Prime KL, Whitesides GM. Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide) – a model system using self-assembled monolayers. J Am Chem Soc 1993;115:10714–21.
[29] Zhang MQ, Desai T, Ferrari M. Proteins and cells on PEG immobilized silicon surfaces. Biomaterials 1998;19:953–60.
[30] Liang J, Rosa LG, Scoles G. Nanostructuring, imaging and molecular manipulation of dithiol monolayers on au(111) surfaces by atomic force microscopy. J Phys Chem C 2007;111:17275–84.
[31] http://www.rcsb.org.
[32] Hu X, Viesselman C, Nam S, Merriam E, Dent EW. Activity-dependent dynamic microtubule invasion of dendritic spines. J Neurosci 2008;28:13094–105.
[33] Dent EW, Kwiatkowski AV, Mebane LM, Philippar U, Barzik M, Rubinson DA, et al. Filopodia are required for cortical neurite initiation. Nat Cell Biol 2007;9:1347–59.
[34] Case MA, McLendon GL, Hu Y, Vanderlick TK, Scoles G. Using nanografting to achieve directed assembly of de novo designed metalloproteins on gold. Nano Lett 2003;3:425–9.
[35] Hu Y, Das A, Hecht MH, Scoles G. Nanografting de novo proteins onto gold surfaces. Langmuir 2005;21:9103–9.
[36] Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric control of cell life and death. Science 1997;276:1425–8.