Self-Aligned Functionalization Approach to Order Neuronal Networks at the Single-Cell Level

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ABSTRACT: Despite significant progress, our knowledge of the functioning of the central nervous system still remains scarce to date. A better understanding of its behavior, in either normal or diseased conditions, goes through an increased knowledge of basic mechanisms involved in neuronal function, including at the single-cell level. This has motivated significant efforts for the development of miniaturized sensing devices to monitor neuronal activity with high spatial and signal resolution. One of the main challenges remaining to be addressed in this domain is, however, the ability to create in vitro spatially ordered neuronal networks at low density with a precise control of the cell location to ensure proper monitoring of the activity of a defined set of neurons. Here, we present a novel self-aligned chemical functionalization method, based on a repellant surface with patterned attractive areas, which permits the elaboration of low-density neuronal network down to individual cells with a high control of the soma location and axonal growth. This approach is compatible with complementary metal-oxide–semiconductor line technology at a wafer scale and allows performing the cell culture on packaged chip outside microelectronics facilities. Rat cortical neurons were cultured on such patterned surfaces for over one month and displayed a very high degree of organization in large networks. Indeed, more than 90% of the network nodes were settled by a soma and 100% of the connecting lines were occupied by a neurite, with a very good selectivity (low parasitic cell connections). After optimization, networks composed of 75% of unicellular nodes were obtained, together with a control at the micron scale of the location of the somas. Finally, we demonstrated that the dendritic neuronal growth was guided by the surface functionalization, even when micrometer scale topologies were encountered and we succeeded to control the extension growth along one-dimensional-aligned nanostructures with sub-micrometrical scale precision. This novel approach now opens the way for precise monitoring of neuronal network activity at the single-cell level.

INTRODUCTION

Because of the complexity of the brain structure and the difficulty of access linked to the presence of many non-neural cells and the vasculature, it is very difficult to isolate the analysis of neuronal function in vivo. In contrast, a very sharp level of analysis can be achieved by in vitro systems, notably when working at the single-cell level. A better understanding of neuronal communication at the level of small neuronal networks actually represents a fundamental step toward a better description of overall neuronal function. Although such systems are simplified in comparison with in vivo neural networks, it was shown that isolated neurons, for which axonal growth has been oriented remarkably, retain their physiological, physical, and dynamic transport properties. Consequently, finding new methods at the single-cell level aiming at spatially tracking propagation of action potentials fired by a defined set of neurons is a very active field of research. In this context, the miniaturization of electronic components, together with the emergence of nano-biotechnology coupled with the mastering of ordered cultures holds great promise to get further insight on neuronal function at the single-cell level. Several types of nanodevices have been proposed to increase the recording resolution of neuronal activity. One of the main issues of interfacing these nanodevices with ordered cultures is, however, the difficulty to maintain neurons alive when cultured at low density and to limit the potential parasitic impact of glial cells during recordings. Although some works succeeded in culturing hippocampal or cortical neurons at low density for several weeks, the patterning of low-density neuronal networks down to single neurons with a perfect control of the localization, together with its interfacing with recording capabilities has not been satisfactorily achieved to date (Table 1).

To create spatially ordered neuronal networks by controlling the location of somas and their extensions, two main approaches have been proposed. First, the positioning of...
somas and the orientation of dendritic growth can be forced by surface topographic structuration, where mechanical constraints impose the organization of the cellular network. The surface structuration can be done by SU8 channels and holes that trap somas and guide neurite growth \(^14,15\) or by nanostructures patterned on the chip surface, such as nanopillars, \(^16\) mushrooms, \(^17,18\) microtubes, \(^19,20\) or scaffolds. \(^21\) Although these topographic strategies indeed allow an efficient control of network architecture, they suffer from fabrication compatibilities when coupled with the micropatterning of recording devices.

The second approach to create ordered neuronal networks is based on chemical surface functionalization. In most cases, attractive areas are created by adhesion molecules, such as laminin, poly-ornithine, poly-ethylenimine, or poly-lysine, which favor cellular adhesion on the substrate. In some cases, they can be associated with repulsive areas coated with repellant functionalization (poly(ethylene glycol) or hydrophobic treatment), thereby allowing the control of neuronal growth. \(^3,22−28\)

This approach has been widely used in the 90’s and early 2000’s and was based on microlithography \(^22,29\) or microcontact printing \(^25,30−35\) and allowed localized neuronal attachment and control of extension growth on large micropatterns. However, the recent emergence of nano-biodevices highlighted a remaining major and still unresolved challenge, i.e., to improve both the cell location and the rate of spots occupied by a single soma to efficiently couple such ordered neuronal networks with nanodevices for electrical activity monitoring. \(^5,7,23,36,37\) Indeed, microcontact printing presented intrinsic limitations due to its alignment precision (500 nm). Likewise, conventional microlithography based on resist structuration suffered from the interdependence between microfabrication and biological analysis (Figure 1a), thereby introducing serious drawbacks, such as degradation of the adhesion molecule during the resist removal or the limited time allowed between the different procedures.

To address these different issues, we propose here a new robust, “self-aligned” chemical functionalization approach at the wafer scale, which aims at precisely locating neuronal bodies and guiding the growth of their extensions. This strategy, which is easier and less constraining to process than the conventional ones, allows creating ordered neuronal networks with a very high rate of perfectly located single cells and a precise location of axonal growth with a sub-micrometer scale precision, as well as an improved survival rate. Moreover, the complete dissociation between microfabrication and biological processes allows performing the biological assays out of the clean room facilities, further improving the quality and flexibility of functionalization steps.

### RESULTS AND DISCUSSION

To create ordered neuronal networks and to selectively localize neuronal attachment, the classical approaches usually perform conventional photolithography to pattern hydrophobic (repulsive) and hydrophilic (attractive) zones. From a technological point of view, the photolithography step is performed after the hydrophobic treatment. However, because of the weak resist adhesion on such modified surfaces, the hydrophobic properties of the surface must be subsequently degraded to enhance the adherence of the resist to the substrate, \(^23\) leading to a decrease of the contrast between zones with and without neuronal growth. In contrast to these conventional approaches that use a topographic resist patterning to localize the adhesion molecule, our self-aligned functionalization (SAF) approach is based on a very high hydrophilic/hydrophobic surface contrast that promotes a very precise control of the positioning of adhesion molecules.
In a first step, the micropatterns are defined by photolithography on a silicon dioxide surface thermally grown on a (100) silicon substrate. The substrate surface is coated with a photoresist and insolated with i-line photorepeater (stepper CANON FPA 3000i4) to allow a large-scale fabrication (6 in. wafer) and a high pattern resolution (critical dimension: 350 nm). Next, the hydrophobic treatment is performed using a vapor deposition technique with a surface-prepared deposition (SPD) equipment (Memsstar) to create a hydrophobic self-assembled monolayer (SAM) on areas not covered by the resist. The resist can then be stripped by washes in acetone and piranha solution (H2O2/H2SO4, 1:1) to obtain a very high contrast between hydrophilic and hydrophobic areas. Finally, the molecules allowing cell adhesion are selectively coated on the hydrophilic micropatterns previously covered by the resist. Thanks to the SAF process, the selective coating with the adhesion molecules is based on high hydrophobic/hydrophilic contrast, contrary to conventional approaches where the coating is controlled by the structuration of the resist. By avoiding resist adhesion issues on hydrophobic surfaces, a very high level of repellant treatment can be used outside the patterned network. Moreover, a complete dissociation between microfabrication and biological steps is obtained, allowing to perform the coating step with adhesion molecules only when needed for the neuronal culture (Figure 1a), without any additional step in clean room facilities.

One of the key steps of this procedure is to ensure that the hydrophobic treatment of the substrate is as efficient as the topological structuration induced by the resist masking to ensure the subsequent selective coating of the adhesion molecules on the hydrophilic surface. Commonly, hydrophobic treatment is achieved using a liquid-phase SAM, grown with a chlorosilane-based precursor. However, this approach presents some drawbacks, such as complex process control, possible risks of contamination of the substrate during the wet chemical process, or run-to-run reliability issues with large variability of SAM coating efficiency. As a reference, we have reproduced a liquid-phase process similar to what was described in refs, leading to a measured contact angle of 60 ± 1°. In addition, the stability of the passivation was very weak, as we observed a very quick degradation upon acetone or piranha washes (Figure 1b). To overcome these issues, we used a vapor deposition treatment (SPD) with 1H,1H,2H,2H-perfluorodecyltrichlorosilane (FDTS). This vapor phase approach demonstrated a very high hydrophobic surface (contact angle of 110 ± 1°) at a large scale (6 in. wafer).

Figure 1. (a) Comparison between the conventional chemical treatments used to order neuronal networks (1) and our new self-aligned functionalization (SAF) approach (2) that allows a complete dissociation between microfabrication and biological assays. (b) Measurement of time and chemical stability for the hydrophobic liquid-phase treatment and the vapor phase treatment with the surface-prepared deposition (SPD) device. (c) Fluorescent images of several patterned surfaces (either large gear wheel patterns, left, and narrow 1 μm wide lines, right) treated with our new SAF procedure and coated for 5 h with fluorescein isothiocyanate (FITC)-labeled poly-L-lysine. Scale bars are, respectively, 300 and 100 μm.

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with a long-lasting stability (more than 2 months). It equally exhibited high chemical stability, with no degradation even after washes in acetone or piranha solutions (Figure 1b), while avoiding issues previously mentioned, such as lack of reliability, contamination issues, or process difficulties.39,40

To validate this approach, we visualized the deposition of fluorescein isothiocyanate (FITC)-labeled poly-L-lysine after 5 h of coating to ensure the uniformity of deposition of this adhesive molecule (see the Supporting Information). As expected, poly-L-lysine only stuck on hydrophilic surfaces. We observed high selectivity and resolution, with a control of the location of the adhesion molecule at the micron scale, even when using complex designs (Figure 1c). Next, we investigated the growth of neuronal cultures on such patterned surfaces. We used cortical neurons prepared from embryonic Sprague-Dawley rats at gestational day 18. Our protocol aimed at minimizing the number of glial cells present in the culture (elimination by density gradient and culture in serum-free medium) since they would divide after plating and thus perturb the creation of well-defined neuronal networks. We designed a micropattern network, composed of large spots for soma settling that were connected with lines to guide neurite growth (Figure 2a). For the biological assays, to favor cell adhesion, the chips were functionalized using an overnight coating based on two complementary adhesion molecules (laminin and polyornithine).41,42 We used laminin because it is an efficient adhesion molecule, representing one of the main extracellular matrix proteins found in the brain and because many studies demonstrated its benefit to promote neurite elongation and differentiation.35-37 Because laminin cannot graft directly on a SiO2 surface, we first applied a poly-ornithine coating.45 Neurons were plated immediately after dissection and maintained in serum-free Neurobasal medium (see details in the Supporting Information Section) with 5% of CO2 and at 37 °C.46-50 A few hours after plating, we observed cell adhesion by sedimentation on the substrate. When neurons were located on hydrophobic surfaces free of adhesion molecules, they either migrated to hydrophilic areas or died by apoptosis. In contrast, neurons readily settled onto coated hydrophilic surfaces and started to extend projections. As a result, well-ordered neuronal networks were successfully created, with somas being positioned on the nodes of the network and neurites connecting each node with a very good level of organization in large networks, even when culture was performed at very low density (Figure 2b-e). After 3 days in vitro (DIV), more than 90% of network nodes (400 μm² spots) were settled by at least one soma and almost 100% of the connecting lines were occupied by a neurite, with a very good selectivity (low parasitic cell connections). As shown in Figure 2d, when using a pattern similar to the laboratory (LAAS) logo, more complex designs demonstrated the high ability of the SAF approach to guide extension growth along the functionalized lines.

We next sought to improve the survival of our ordered neuronal networks cultured at low density, even after neurons had maturated (10 DIV). To this aim, different approaches have been described, such as covering the neuronal culture with...
a separate glass coverslip supporting a culture of glial cells, to be used as a feeding layer. Rather, we chose an easier-to-implement approach, on the basis of the use of conditioned medium derived from glial cells. To evaluate the impact of the culture medium on the metabolic activity of neurons, we used a colorimetric assay, i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (shown in the Supporting Information). We first plated cortical neurons on several flat surfaces (i.e., silicon, silicon oxide, and aluminum) and cultivated them in serum-free Neurobasal medium, and we used a reference well of neurons grown directly on plastic without any chip. Although the measurement of the metabolic activity proved the biocompatibility of all materials at 3 DIV (>80% compared with the reference), it also revealed a significant decrease (of at least 70%) of neuronal metabolic activity from 3 to 9 DIV. In sharp contrast, when the medium was replaced by medium conditioned by glial cells, the mitochondrial activity of neurons rose drastically (+730% at 9 DIV compared with the reference at 3 DIV). On the basis of these findings, we thereafter systematically replaced the culture medium.

Figure 3. (a) Graph describing the impact of the neuronal concentration on the occupation rate of the spots of the network, as well as on the rate of spots bearing a single soma. (b) Graph showing the impact of the spot size on the number of somas per spot for a density of 200 cells/mm². (c, d) Comparison between two sizes of spots, 400 μm² (c) and 78 μm² (d), performed by immunofluorescence and confocal microscopy, illustrating nuclei labeled with DAPI (blue), dendrites labeled with MAP-2 (red), and axons labeled with TAU (green). (e–f) Immunofluorescence confocal images of two widths of connecting lines (e) 1 μm and (f) 10 μm and nuclei labeled with DAPI (blue), dendrites labeled with MAP-2 (red), and axons labeled with TAU (green). Scale bars are 20 μm for all pictures.
medium after 3 DIV by a conditioned medium derived from glial cells, to achieve long term survival of low-density neuronal networks. The Figure 2f shows a fluorescent picture of a mature 15 DIV neuronal network after a viability assay (calcein AM/ethidium homodimer), further proving that cells indeed remained healthy, even when cultured at low density.

We next assessed the impact of two different parameters to improve both the positioning of somas down to a single cell and the organization of the neuronal network. All statistics presented in this article were performed using three independent sets of cultures, overall including more than 2000 spots.

First, we evaluated the influence of the cellular concentration using three different densities (from 500 to 200 cells/mm²), on the same spot size (square of 400 μm²), and under the same culture conditions. It appeared that a lower occupation rate was observed when the cell concentration decreased, dropping from 92% for concentrations of 500 cells/mm² down to 60% for 200 cells/mm². However, the lowest concentration was also found to be more favorable to maximize the unicellular character. Indeed, the rate of occupied spots bearing a single cell increased from 17.5 to 41% when the cell concentration was lowered from 500 to 200 cells/mm² (Figure 3a). Nevertheless, when the cellular density became too low (<200 cells/mm²), we noted that it severely compromised the quality of neuronal growth and no network could thus be formed.

In parallel, we analyzed the influence of the design patterns. We reasoned that a reduction of the spot size could improve the positioning of somas as well as increase the unicellular rate. Figure 3b presents the occupation rate for four different sizes of a spot (S = 400 μm², S = 314 μm², S = 154 μm², and S = 78 μm²). Even if the occupation rate logically decreased with the spot size, when considering the occupied spots, we noted that the rate spots occupied by only one neuron strongly increased, from 41% with 400 μm² spots to 74% with 78 μm² spots.

Finally, we combined the two previous findings and sought to pattern selective low-density neuronal networks characterized by a maximal single-cell occupancy rate at a defined place by using both the smallest size of spots (78 μm²) and a low cell density (200 cells by mm²). This allowed the generation of very well-ordered networks, with almost 75% of occupied spots bearing a single cell (Figure 3c,e) and where all somas were interconnected. As a matter of fact, the location of the neurons could be perfectly mastered because the chosen size spot is in the range of the size of a soma.

We next applied a similar strategy to control growth of neuronal extensions, using several widths of lines (patterned from 10 to 1 μm) connecting the soma spots, to impose the growth track of the neurites. As shown in Figure 3f, a 10 μm wide line displayed multiple neurites distributed on the same track. In contrast, when the line width was in the same scale (∼1 μm) as the neurite itself (Figure 3e), we could evidence that a single extension was growing on each functionalized line. As a result, our self-aligned functionalization approach allowed creating neuronal networks with a very high degree of organization, a very precise control of both the location of the somas and growth of neuronal projections, at the single-cell level.

Finally, we evaluated the behavior of SAF on structured surfaces to further demonstrate its potentialities

(i) from a phenomenological point of view, i.e., to test whether surface functionalization could still impose the direction of neurite growth even in the presence of topological obstacles and,

(ii) from a technological point of view, i.e., to assess whether it was possible to precisely align such ordered networks on nanodevices to record neuronal activity (planar nanoparticle field extraction thruster, vertical nanoprobe).

To this aim, we cultured neurons on chemically functionalized patterns aligned on micrometer scale topologies (Figure 4a). Interestingly, we demonstrated that the growth of neuronal extensions was still guided by the chemical functionalization even when micrometer walls were encountered (Figure 4b,c), proving that our surface chemical treatment is predominant over topographic structuration. Because our approach uses standard complementary metal-oxide–semiconductor protocols at the wafer scale, it could beneﬁt not only from processes with nanometer-scale resolution in structure patterning but also in terms of multilayer mask alignment.
that scope, the SAF was aligned on multiple vertical one-dimensional (1D) nanostructures (diameter of 200 nm and height of 3 μm) using a standard i-line photorepeater, as shown in Figure 4d. The 1 μm wide functionalized line was able to guide growth of neurites along these structures, with a perfect control of location at a micrometrical scale, thereby promoting a large interaction between nanowires and neurites (Figure 4e).

Table 1 displays a comparison of the self-aligned functionalization technique with other approaches from the literature\(^ {23,25,29,34,35} \) to pattern a neuronal network on an electronic device. This synthetic benchmark highlights the benefits of the self-aligned functionalization strategy at a large scale with a precise control (sub-micrometre scale) of cell location and dendrite extension at the single-cell level. This approach now opens the opportunity of implementation with new 3D nanodevices\(^ {16,7,29,60} \) for neuronal interfacing, which has emerged in the last few years and requires high alignment precision (deca-nanometer scale) on tridimensional nanostructures in comparison with the conventional methods.

It also opens the way for new perspectives regarding the in vitro monitoring of neuronal activity since it allows both the refinement of the analysis down to single cells connected in networks, together with the capacity to stimulate and record the cell somas, as well as to track the signal along the axon, as highlighted in the Figure 4f.

### CONCLUSIONS

In summary, we have designed an innovative and robust self-aligned functionalization strategy, allowing ordering neuronal networks at the single-cell level with unparalleled precision. This approach presents improved time and chemical stabilities, together with a complete dissociation between microfabrication and biological assays. Wide neuronal networks at low density were obtained by this method, with a very good selectivity (low parasitic cell connection). By tuning the conditions of cell growth and the design of the patterns, we achieved the construction of neural network with a very high rate of unicellular spots (75% on occupied spots) connected by single neurite extensions. Decreasing the spot size in our patterns improved our control of the location of the somas, resulting in neuronal networks at the single-cell level with a micrometrical control of positioning. Finally, we proved that our SAF approach could efficiently guide neurite outgrowth even when micrometre scale topologies were encountered, allowing to precisely control neuronal extension along aligned 1D nanostructures with sub-micrometrical scale precision.

### METHODS

**Process of Self-Aligned Functionalization to Obtain Hydrophobic Areas with FDTS and Hydrophilic Areas Coated with Adhesion Molecule.** Silicon substrate (100) p-type (2–10 Ω cm) was cleaned by oxygen plasma in a TEPLA machine (800 W, 5 min). Then, a photoresist ECI 3012 1.1 μm was spin-coated on the substrate and exposed with a CANON stepper to de

**Photolithography.** Photolithography was performed on an ECI-coated (thickness = 1.1 μm) silicon substrate with a dose of 750 mJ/cm\(^2\) and a numerical aperture of 0.63. Then, the sample was post-exposure-baked at 110 °C during 60 s, developed in an ECI developer (MF-CD-26) during 35 s, and rinsed in DIW using an automated resist processing system (EVG 120). Resist nanopatterns were obtained with a diameter of 400 nm, and this diameter could be reduced and tuned using a short plasma O\(_2\) step.

Then, the vertical resist nanopillars were transferred onto the substrate by reactive ion etching based on SF\(_6\) (45 sccm), CF\(_3\) (30 sccm), and O\(_2\) (2 sccm) to ensure an anisotropic etching. The residual ECI layer was finally removed by plasma O\(_2\) (800 W, 15 min) and washed in piranha solution (H\(_2\)O\(_2\)/H\(_2\)SO\(_4\), 1:1) during 5 min, rinsed in DIW, and dried using a spin coater under N\(_2\) flow.

**Culture of Rat Cortical Neurons.** Primary cortical neurons were prepared from Sprague-Dawley rat embryos at gestational day 18 using a previously described procedure\(^ {48,61} \) with the following modifications: after dissection, the cortex tissue was dissociated by incubation for 15 min at 37 °C in phosphate buffer saline (PBS) containing 10 U/mL Papain (Worthington), followed by gentle dissociation in PBS containing 1.5 mg/mL bovine serum albumin (BSA) and 1.5 mg/mL Trypsin inhibitor (from chicken egg, Sigma). After dissociation, cell homogenates were subjected to a centrifugation step at 3000g for 10 min through a 4% BSA cushion (diluted in Neurobasal medium), to minimize presence of glial cells in the final pellet. Cultures were maintained in serum-free Neurobasal medium (Invitrogen) supplemented with 0.5 mM glutamine, 1% penicillin/streptomycin, and 2% B-27 supplement (Invitrogen). Cells were plated on the chips at a concentration between 200 and 500 cells/mm\(^2\) and cultivated in a humidified incubator at 37 °C and 5% CO\(_2\).

**Viability Assay.** This viability assay (Live/Dead, Thermofisher Scientific) allows discriminating live from dead cells. Indeed, the polyanionic dye calcein AM is retained within live cells and produces a green fluorescence, whereas ethidium homodimer-1 (EthD-1) only enters cells with damaged membranes and produces a red fluorescence in dead ones. Rat cortical neurons were plated on chemically functionalized samples in serum-free Neurobasal medium. After 3 days in vitro (DIV), this medium was replaced by medium conditioned by glial cells. At 15 DIV, each sample was rinsed with 1 mL of Dulbecco’s phosphate-buffered saline before being covered by the Live/Dead reagent (calcein AM 1 μM: EthD-1 1 μM in serum-free Neurobasal medium minus phenol red). Then, samples were incubated for 20 min at 37 °C and observed with a fluorescence microscope.

**Preparation of Conditioned Medium by Glial Cells.** After dissection, each cortex hemisphere was cut into small pieces, placed into a 50 mL Falcon tube, and digested with papain (4.3%) at 37 °C for 8 min. Then, papain was inactivated with Lo-Ovo (ovomucoid solution) and tissues were dissociated by vigorous pipetting using a plastic pipette (10–20 times). The resulting mixture was filtered with a 70 μm cell strainer and subjected to a centrifugation step at 1100g for 10 min. The cell pellet was resuspended in Dulbecco’s modified Eagle’s medium supplemented with 10% of fetal calf serum and 1% penicillin/streptomycin and plated in a cell culture flask. After 10 days, this medium was replaced by serum-free Neurobasal medium (Invitrogen) supplemented with 0.5 mM glutamine, 1% penicillin/streptomycin, and 2% B-27 supplement (Invitrogen). Three days later, this conditioned medium was harvested to be used for neuronal culture.

**Immunofluorescence and Confocal Microscopy Analysis.** Samples were rinsed with PBS, fixed with 4% paraformaldehyde for 30
min, and rinsed again twice with PBS. The cells were incubated for 4 min in permeabilization buffer, 0.1% Triton X100 in PBS, and rinsed with PBS/0.02% Tween 20. Then, they were incubated for 15 min in blocking solution, 2% BSA in PBS/0.02% Tween 20. The primary antibodies MAP-2 and TAU were applied for 1 h at room temperature in a humidified chamber (Anti-TAU (1/500), Anti-MAP-2 (1/250), Millipore) and rinsed 3 times for 3 min with PBS/0.02% Tween 20 while shaking. The secondary antibodies conjugated with Alexafluor-488 or Alexafluor-594 (Millipore) were applied for 1 h at room temperature in a humidified chamber and rinsed 4 times for 3 min with PBS/0.02% Tween 20 while shaking. The cells were incubated for 4 min in counterstaining solution: DAPI 0.02 mg/mL in PBS Tween with PBS/0.02% Tween 20 while shaking. The cells were incubated for 4 min in medium (Molecular Probes), and we used a coverslip to seal the dehydrated by successive incubations in graded ethanol washes (25, 4 h. Then, glutaraldehyde was rinsed with PBS and samples wereblocked solution, 2% BSA in PBS/0.02% Tween 20. Then, they were incubated for 15 min in blocking solution, 2% BSA in PBS/0.02% Tween 20. The primary antibodies MAP-2 and TAU were applied for 1 h at room temperature in a humidified chamber (Anti-TAU (1/500), Anti-MAP-2 (1/250), Millipore) and rinsed 3 times for 3 min with PBS/0.02% Tween 20 while shaking. The secondary antibodies conjugated with Alexafluor-488 or Alexafluor-594 (Millipore) were applied for 1 h at room temperature in a humidified chamber and rinsed 4 times for 3 min with PBS/0.02% Tween 20 while shaking. The samples were placed on a mounting medium (Molecular Probes), and we used a coverslip to seal the samples with nail polish.

Preparation for SEM Imaging. For the SEM imaging, cultures were rinsed with PBS and fixed with 5% glutaraldehyde in water during 4 h. Then, glutaraldehyde was rinsed with PBS and samples weredehydrated by successive incubations in graded ethanol washes (25, 50, 80, and 100%) each time during 8 min. Samples were dried and images made with a FEI Helios 600i SEM.

■ ASSOCIATED CONTENT
2 Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.8b00529.

MTT assays; impact of coating duration on the uniformity of deposition of the adhesion molecule (PDF)

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The authors declare no competing financial interest.

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