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Agarose-Based Substrate Modification Technique for Chemical and Physical Guiding of Neurons In Vitro

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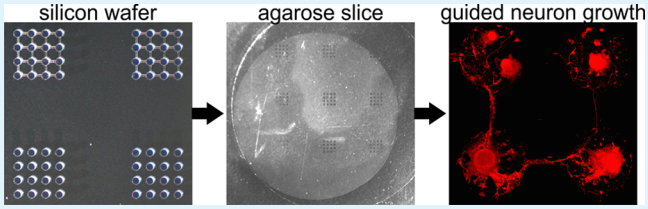
ABSTRACT: A new low cost and highly reproducible technique is presented that provides patterned cell culture substrates. These allow for selective positioning of cells and a chemically and mechanically directed guiding of their extensions. The patterned substrates consist of structured agarose hydrogels molded from reusable silicon micro templates. These templates consist of pins arranged equidistantly in squares, connected by bars, which mold corresponding wells and channels in the nonadhesive agarose hydrogel. Subsequent slice production with a standard vibratome, comprising the described template pattern, completes substrate production. Invertebrate neurons of locusts and pond snails are used for this application as they offer the advantage over vertebrate cells as being very large and suitable for cultivation in low cell density. Their neurons adhere to and grow only on the adhesive areas not covered by the agarose. Agarose slices of 50 μm thickness placed on glass, polystyrene, or MEA surfaces position and immobilize the neurons in the wells, and the channels guide their neurite outgrowth toward neighboring wells. In addition to the application with invertebrate neurons, the technique may also provide the potential for the application of a wide range of cell types. Long-term objective is the achievement of isolated low-density neuronal networks on MEAs or different culture substrates for various network analysis applications.



KEYWORDS: *hydrogel, defined network, patterning, vibratome, nonadhesive, invertebrates*

1. INTRODUCTION

The understanding of the development and function of neuronal networks is of high interest but at the same time a big challenge. The investigation of neuronal networks in vitro helps to overcome the highly complex situation in vivo. However, also in in vitro approaches simplification of complex networks seems to be necessary for a detailed understanding of network processing.

This simplification could be achieved by creating defined small networks in vitro. In really small networks with less than 20 neurons, individual cells can be identified and a better overview of the activity of single units and their influence on the functionality of the network can be established.[1,2] These conditions can easily be achieved by using invertebrate neurons. Network activity in vitro can be investigated with different methods. Meanwhile, noninvasive electrophysiological methods are state of the art as they allow for long-term observations of neuronal activity. A common method here is the use of multielectrode arrays (MEAs),[3−5] but other extracellular recording electrodes[6−8] and optical methods such as Ca^{2+}-imaging[9,10] are also used.

The development of small and defined neuronal networks requires patterning of the growth substrate. Patterning means subdivision of the substrate with special chemical coatings and/or topographically raised barriers into cell-attracting regions and regions where cell adhesion and neurite outgrowth are inhibited. Over the years, different technologies to achieve such network patterns were developed ranging from printing, adsorption, photo- and soft-lithography to laser writing or combinations of these.[7−24] More recent studies even used magnetic[25] cues and electrophoresis[26] in addition to traditional pattern technology to improve neuron positioning. However, all these and related studies focus on the specific demands of the applied cell system to its microenvironment (for reviews see ref 27).

A widely used patterning substance is agarose. Agarose is a natural, noncytotoxic, and transparent hydrogel allowing easy conversion from gel to liquid. Agarose is nonadhesive for many cell types. Thereby, agarose substrates may provide a chemical as well as physical barrier for cells in culture.[9,15,16,19,28−32] Agarose patterning can be used for the positioning of single cells but also for isolation of small cell clusters. Moreover, agarose patterned small networks on electronic devices or other substrates can be utilized for neuronal activity investigations[9,16,19,31,33] with various methods.

In this study, a new and simple method for the fabrication of agarose substrates patterned by soft-lithographic techniques is presented. We used photolithographic microstructured templates for molding defined topologies in agarose. The final product consists of thin agarose slices of defined thickness cut

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by a vibratome and containing open wells and closed channels which are positioned on a variety of substrates. The vibratome slices were tested for patterning of cultures of two different invertebrate biological test systems. Neurons of the locust *Locusta migratoria* and of the pond snail *Lymnaea stagnalis* were used. Insect and mollusk neurons provide the advantage over vertebrate cells of their suitability for the building of small networks in vitro as they can be cultured in low cell density.[1,2,34−36] Furthermore, many neurons of invertebrates are very large (>100 μm), so they can cover individual electrodes of a MEA completely which leads to a better signal-to-noise ratio. In addition, a one-to-one coupling of neuron and electrode in such networks is attractive as, like this, the unambiguous allocation of signals to certain cells can be done. Nevertheless, the presented slices for cell growth guiding produced by our technique provide the potential for the application of a far wider range of cell systems, which include cells from vertebrates.

2. EXPERIMENTAL SECTION

2.1. Template Design. The template devices consisted of either 4 × 4 arrangements of 16 circular pins (Figure 1a, c) or 8 × 8 arrangements of 60 circular pins (Figure 1b). The pins were either completely isolated from each other or connected by low bars. Each pin molded a corresponding hole in an agarose hydrogel and each bar molded a low channel. Pin diameter, pin interspace, and the length and number of bars varied among different arrangements to either suit different MEA layouts or to test different conditions for cultured neurons. Detailed parameters can be obtained from Figure 1. Onto each 16-pin arrangement with bars two triangular shapes were added. These triangles, after cutting slices, helped to choose the correct orientation of the agarose slice with the channels pointing toward the substrate as the channels were open only to one side of the subsequent slice. The orientation was correct when the acute angles pointed clockwise.

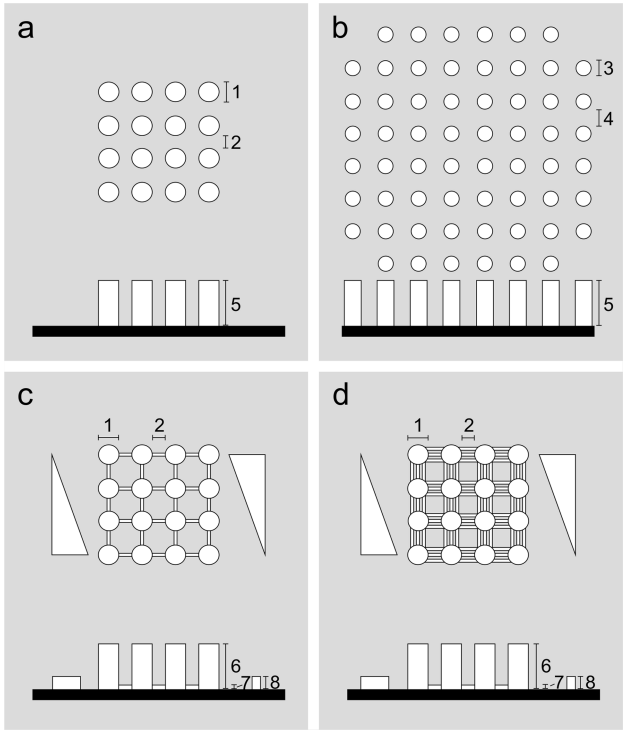

Figure 1. Schematic illustration of silicon template geometries and dimensions as top and side view: (a) 16-pin arrangement without bars; (b) 60-pin arrangement without bars; (c) 16-pin arrangement with pins interconnected by single bars and triangles for agarose slice orientation; and (d) 16-pin arrangement with pins interconnected by triple bars and triangles for agarose slice orientation. Dimensions (not true to scale): pin diameter (1) 110 μm; pin interspace (2) 90 μm; pin diameter (3) 90 μm; pin interspace (4) 110 μm; pin height (5) 250 μm; pin height (6) 180 μm; bar height (7) 5 μm; triangle height (8) 15 μm.

2.2. Photolithography. The templates described above were fabricated on a silicon wafer with titanium undercoating by photolithographic techniques (Figure 2).[37,38] Pretreatment with oxygen plasma (3 min; 300 W; 200 sccm of O_2) and 10 min at 120 °C on a hot plate was done before the wafer was spin-coated with an UV-curable epoxy-based photoresist (SU-8 5 and SU-8 100, Microchem Corp, Newton, MA). The desired structure heights were achieved in a two-step coating process: one for the bar height and one for the pin height using SU-8 5 and SU-8 100, respectively. After each spin-coating process a relaxation time at room temperature followed. Then, soft baking of the deposited layer was performed with appropriate temperature profiles including temperature ramps to avoid film stress and cracks. Subsequently, exposure of the layer was achieved using UV light and the corresponding mask. Afterward, a post-exposure bake to increase cross-linking and to stabilize the irradiated areas of the resist was performed. Finally, the wafer was developed in mr-DEV 600 (micro resist technology, Berlin, Germany) followed by rinsing steps with isopropanol and water. Several hundreds of arrangements with the described dimensions were arrayed on one silicon wafer (Figure 2a).

2.3. Agarose Slice Fabrication. To produce agarose gel slices with guiding structures, an agarose stock solution of either 3% or 4% agarose (NEEO ultraquality, Carl Roth, Karlsruhe, Germany) in phosphate buffered saline (PBS, Sigma-Aldrich, Steinheim, Germany) was prepared and sterilized by autoclaving (121 °C, 20 min). Both concentrations were equally suitable for the application. Lower and higher agarose concentrations resulted in either instable or brittle gel layers. The cured agarose stock solution could be reused several times by warming in a microwave oven. The following procedure is shown schematically in Figure 3: The wafer with the templates was preheated on a hot plate (50 °C) to avoid fast hardening of the gel. Liquid agarose was cast over the silicon wafer until the pins were fully covered. The culture dish with the wafer was panned to get an equal distribution of the gel over the whole wafer and to prevent the inclusion of air bubbles between the agarose and the wafer surface. The wafer was transferred to a refrigerator where the agarose solidified in a couple of minutes. Afterward, the hardened replica on the wafer surface, about 0.5 cm thick, was peeled off. Agarose gel cylinders for cultures on glass or culture dishes were punched out such that the bottom part of each cylinder contained several well arrangements. For cultures on MEAs only one arrangement in the middle of the cylinder was taken. The diameters of the cylinders were chosen to suit the growth substrate used for cell culture.

The agarose guiding structures were produced by cutting slices off the cylinders using a vibratome (VT1000 S, Leica Microsystems, Wetzlar, Germany). An agarose cylinder with the well arrangements pointing upward was glued onto the mounting plate of the vibratome which was located in a trough filled with distilled water. The vibration frequency of the vibratome was set to 50 Hz, the cutting velocity was set to 1.6 mm/s, and the thickness of the slices was set to 30, 50, 80, or 120 μm, respectively, to test for slice stability and the influence of the well depth on neurons in culture. In case of well arrangements with channels, naturally, because of the low bar height, only the first slice contained wells and channels. Every further slice still contained isolated wells. According to the pin heights of the wafer, it was possible to produce 3−5 50 μm thick agarose slices per cylinder. After cutting, slices were transferred from the trough to a dish filled with distilled water. Here, they could be stored over a period of at least 8 weeks.

2.4. Culture Substrate Preparation. Agarose slices were positioned on different substrates. We used either glass coverslips (Ø 12 mm, Marienfeld, Lauda-Königshofen, Germany) or polystyrene culture dish surfaces (Ø 35 mm, ThermoScientific Nunc, Braunsch-

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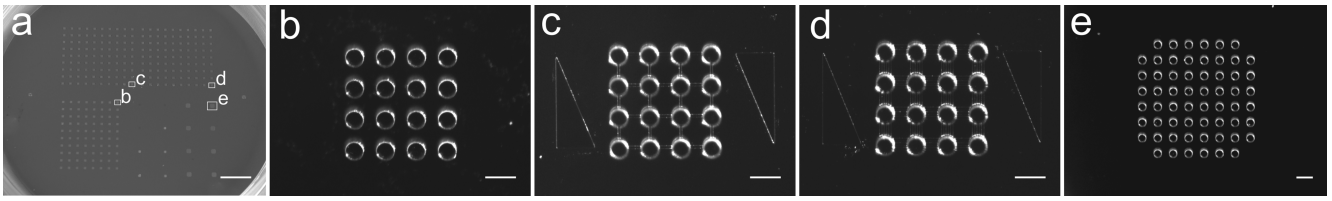

Figure 2. Silicon wafer with pin and bar templates for substrate patterning. (a) Overview showing the arrangement on the wafer of four fields, each consisting of multiples of one of the 4 different pin arrangement designs. Different pin designs as indicated by rectangles in (a) at higher magnification: (b) 16 pins without bar connections, (c) 16 pins with single interconnecting bars, (d) 16 pins with triple interconnecting bars, (e) 60 pins for MEA applications with larger interspace between single arrangements as only one arrangement per MEA and slice was needed. Scale bars: (a) 1 cm, (b–e) 200 μm.

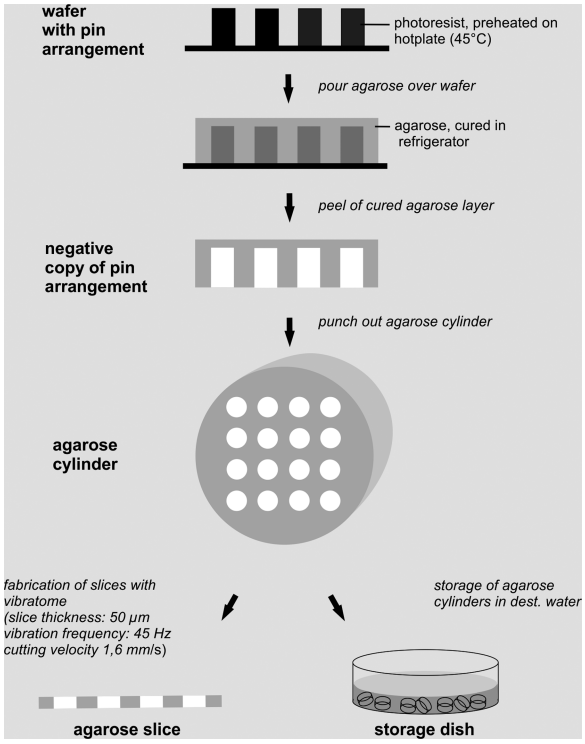

Figure 3. Schematic illustration of agarose slice production process (not true to scale). The silicon wafer was preheated on a hot plate before liquid agarose was poured on the wafer. Agarose curing occurred in the refrigerator within minutes; subsequently, it could be peeled off the template. Cylinders of favored diameter were punched out and either cut into vibratome slices or stored in distilled water for later use.

weig, Germany) as these are used as standard substrates for locust and pond snail cell culture. In addition, the agarose slices were tested on MEA-surfaces, where the electrode material was gold, titanium nitride (Multichannel Systems (MCS), Reutlingen, Germany), or platinum (Qwane Bioscience, Lausanne, Switzerland), and the passivation layer was composed of SU-8 (Qwane Bioscience) or silicon nitride (MCS). Agarose slices for cell culture were sterilized in 70% ethanol for 5 min, subsequently rinsed two times in sterile PBS or distilled water, and then positioned on the substrates. When culturing on glass, coverslips were sterilized by an ethanol flame and positioned in cell culture dishes. Afterward, a droplet of 100 μL of PBS or water was given to the glass coverslips and agarose slices were fixed on them. The correct position of the channels and wells was determined and adjusted under microscopic control. The PBS or water had to evaporate before further steps followed.

2.5. Long-Term Attachment Test. For long-term attachment and stability tests, agarose slices were positioned and culture chambers were filled with cell culture medium and incubated at either 20 or 30 °C without cells. We tested four MEAs and four glass coverslips covered with agarose slices. Four culture dishes with agarose slice covered glass coverslips were placed on a shaking device at room temperature for 3 days, simulating immunohistochemical staining procedure. Attachment was controlled every day. These tests occurred independently from several cell culture experiments with agarose slices, where slice adhesion usually was maintained within the time period of the cell culture experiments which generally was aborted after about 1 week.

2.6. Determination of Agarose Slice Thickness. To investigate the precision of the vibratome, the thickness of different agarose slices was measured using a confocal laser scanning microscope (TCS SP2, Leica Microsystems, Wetzlar, Germany). For this purpose, the agarose was stained with 1% fluorescent microspheres (FluoSpheres sulfate microspheres, 0.2 μm, yellow–green fluorescent), (Molecular Probes, Life Technologies Invitrogen, Darmstadt, Germany) but otherwise treated as described above. The vibratome was set to cut 50-μm-thick slices. Four slices of different agarose cylinders were produced and positioned on glass coverslips. For each of the four slices, three different 16-well arrangements per slice were chosen and three to four well depths per arrangement were measured. In this way, data of 40 wells were obtained. Confocal Z-stacks of stained agarose slices were collected using a 64× oil immersion objective in combination with an argon/krypton laser (excitation wavelength 488 nm, detection range 500–535 nm). The maximum extension of the Z-stacks served as a measure for the thickness of the slices. The obtained data were averaged.

2.7. Animals. Meso- and metathoracic ganglia of male and female *Locusta migratoria* within 1 week after their final molt were used. They were kept in the department's own crowded breeding colonies under a constant light/dark cycle of 12/12 h and were fed with wheat seedlings and wheat bran. Ganglionic rings of the pond snail *Lymnaea stagnalis* were used. The snails originated from the department's own breeding colony. They were kept in 5-L tanks filled with pond water at room temperature and under a natural light/dark cycle. In the winter months the light cycle was supported by artificial light with a light/dark cycle of 12/12 h. The animals were fed with lettuce and cucumber slices. For preparation, young adults with shell lengths of 2–3 cm were used.

2.8. Cell Culture. Preparation of suspension of neurons of *Locusta migratoria* for cell culture was performed as described previously.[39] A droplet of 100 μL of cell suspension was added to the agarose substrates to plate the cells. Cells dispersed randomly into the wells or outside the wells on the agarose surface. The number of cells within the wells could be increased by either raising the cell density of the suspension or by manually positioning of single neurons into the wells using an eyelash. After plating, the cells were allowed to adhere to the substrate for about 1 h before 3 mL of medium was added to each culture dish. For cultivation, the dishes were maintained at 30 °C. The preparation of snail neurons up to the step after enzymatic treatment essentially corresponds to techniques described previously[40,41] with the following exceptions: instead of using ampicillin for defined medium (DM) solution, a mixture of penicillin (10.000 units/mL) and streptomycin (10 mg/mL) (Sigma-Aldrich, Steinheim, Germany) was used. Preparation of ganglia was carried out in sterile normal saline (NS) instead of antibiotic normal saline (ABS). For the last

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preparation step, realized in DM, further non-neuronal tissue as well as the inner ganglionic sheets were removed and the neurons were mechanically dissociated using fine forceps. The inner neuropile was also removed. Thereby, selection of neurons was not limited to specific types. Rather, every ganglion of the ring was dissociated and all cells were used for cell culture to enlarge cell crop and therefore to enlarge chance of well occupancy with cells. Cells were transferred into a low binding tube (2000 μL, Biozym, Hamburg, Germany) filled with DM by a low binding micropipette (200 μL, Biozym). Further steps correspond to the locust preparation procedure described in 39 after enzymatic treatment. For cultivation, the culture dishes were maintained at 20 °C. Neurite outgrowth and viability of the cells were observed by phase-contrast microscopy (Olympus CKX41, Hamburg, Germany) and documented using a microscope camera (Moticam CMOS 3.0 MP, Motic, Wetzlar, Germany).

2.9. Influence of Agarose Slice Thickness on Cell Survival. For culture condition analysis of neurons in different well depths, locust cell culture was performed with three different agarose slice thicknesses. Therefore, agarose slices with corresponding 4 × 4 arrangements of three different heights, 50, 80, and 120 μm, were produced at the vibratome. For each height condition, four independent cell culture experiments were conducted. Thus, every experiment consisted of 4 culture dishes, one for every substrate height condition and one for control, which contained no agarose slice. The cultured neurons of one experiment derived from meso- and metathorax of two animals which were pooled previously to ensure equal original conditions. The number of living cells within the agarose impressions were counted on day in vitro (div) 1, 2, 3, and 7. The particular results of every condition were averaged.

2.10. Immunohistochemistry. Immunohistochemical staining was done using cell cultures between div 4 and 7, because previous observations showed the maximum of neurite outgrowth in this period. The cells were fixed with 4% formaldehyde (FA, 37% Applichem, Darmstadt, Germany). Locust neurons were specifically marked by an antiserum against horseradish peroxidase (1:10 000) (rabbit-anti-HRP, Sigma-Aldrich, Steinheim, Germany)[42] and visualized with a Cy3-conjugated secondary antibody (1:5000) (goat-antirabbit-Cy3, Jackson Immuno Research, Newmarket, Suffolk, UK). For mollusk neurons no specific marker exists. Staining of cells was achieved by using an antiserum against α-tubulin (1:500) (mouse-antitubulin, Exbio Antibodies, Vestec, Czech Republic). The antibody was visualized with a Cy3-conjugated secondary antibody (1:5000) (goat-antimouse-Cy3, Jackson Immuno Research). After staining, substrates were mounted to glass slides with Mowiol (poly(vinyl alcohol) Sigma-Aldrich) and analyzed with the confocal laser scanning microscope.

3. RESULTS

3.1. Agarose Molding. The templates with the positive relief features (Figure 2) produced perfectly patterned agarose hydrogels. With the vibratome, thin agarose slices containing agarose-free circular wells and closed micro channels of defined dimensions were successfully produced. All different designs tested here produced equally satisfying results. Even the fine geometries of the three parallel micro bars only a few micrometers wide and in very close proximity to each other could be replicated perfectly (Figure 4b).

3.2. Slice Stability, Handling, and Attachment. Agarose hydrogel slices were cut down to a thickness of 30 μm without tearing. Although the slices were thin, they were quite stable and could be transferred easily to different dishes. Positioning of the slices on the substrates worked well and wrinkle formation did not occur. The slices adhered reliably to all tested substrates, glass, cell culture dish, and MEA, without the need of any adhesive substances or bonding pretreatment. Despite this, detachment of agarose slices at the end of an experiment

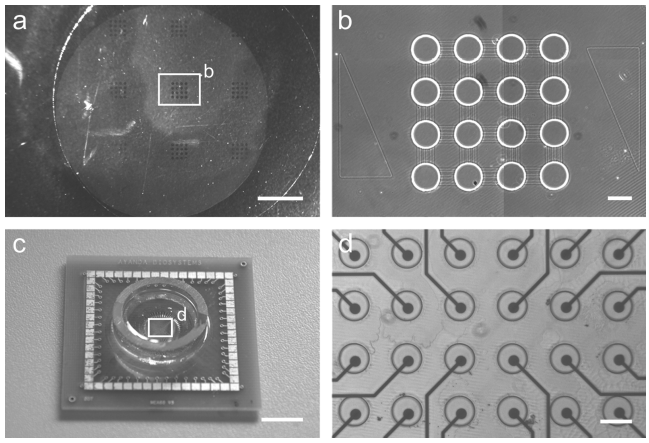

Figure 4. Agarose slice application on glass coverslips and MEA platform. (a) Overview of agarose slice with eight arrangements of 16 well/triple channel connections design on a circular glass coverslip in culture medium. (b) Single arrangement as marked by the rectangle in (a) at higher magnification. (c) MEA device with agarose slice positioned in culture chamber under medium. (d) MEA electrodes in detail with overlying agarose slice (area indicated by rectangle in (c)). Scale bars: (a,c) 1 cm; (b,d) 100 μm.

also proved to be simple, which greatly facilitated the repeated use of agarose slices on standard reusable MEAs.

The time of attachment of the agarose slices to the particular substrates was tested at 20 °C, 30 °C, and room temperature. The results are shown in Table 1. Incubation of agarose slices

Table 1. Long-Term Attachment of Agarose Slices under Corresponding Experimental Conditions

substrate	temperature/condition	days of attachment
MEA1	20 °C/incubator	36
MEA2	20 °C/incubator	20
MEA3	30 °C/incubator	21
MEA4	30 °C/incubator	29
glass1	20 °C/incubator	25
glass2	20 °C/incubator	26
glass3	30 °C/incubator	25
glass4	30 °C/incubator	28
glass5−8	room temperature/shaking device	3 (time for IHC[a])

[a]IHC: immunohistochemistry.

on glass or MEA substrates at 20 and 30 °C led to an attachment time of around 3 weeks. Experiments were aborted after 5 weeks at the latest. The tests on a shaking device at room temperature lasted for 3 days. For optimal attachment of agarose slices, the substrates had to be particularly clean and smooth and agarose slices had to be thoroughly dried after positioning and before medium application, respectively. Furthermore, medium change had to be done very cautiously.

3.3. Slice Thickness. Cells of the locust nervous system were cultivated in patterned agarose slices of different thickness on glass substrate to evaluate the influence of this parameter. Figure 5 shows that the number of cells within the wells in the agarose generally declined over time, whereby the results indicate that cell survival was influenced by the slice thickness, respectively well depth at least from div 7 on. When long-term survival is considered, wells of 120 μm depth showed significant negative effects in comparison to 50 μm deep wells. The best compromise for successful positioning of cells, but also

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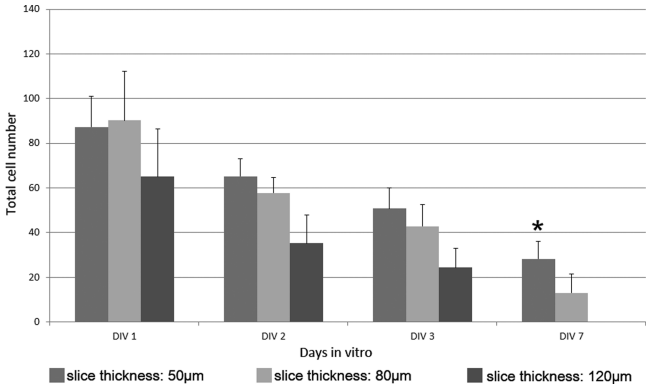

Figure 5. Influence of slice thickness on cell viability over time. Comparison of total cell numbers of *Locusta* in agarose wells of increasing depths within 7 days. Variances on div 1, 2, and 3 could not be determined. The evaluation showed a variance among the 3 conditions only at div 7 (ANOVA, $P < 0.05$). On div 7 cell viability was significantly higher on slices with 50-μm thickness in comparison to slices with 120-μm thickness. * Indicates post hoc Tukey test. (n = 4, independent cell cultures for each slice type).

maintaining cell viability, was achieved with a slice of 50 μm thickness. We also tested whether the vibratome produced such 50 μm sections in a reliable fashion. Measuring the depth of 40 different wells on 11 slices with the confocal revealed an averaged well depth of 58 μm (±5.7 μm). These minor deviations were tolerable for our application.

3.4. Guiding of Cell Growth. Neurite growth was confined to the wells and channels where access to the cell adhesive substrate was provided. Figure 6 shows the development of *Lymnaea* neurons in the wells connected by channels between

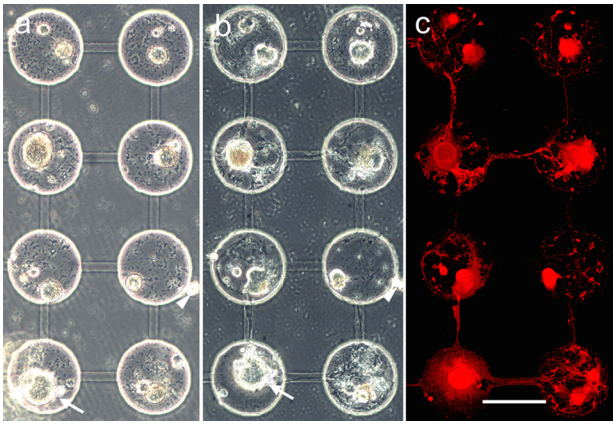

Figure 6. Neural growth within agarose wells (*Lymnaea*). (a) *Lymnaea* neurons positioned in eight wells of a 16-well arrangement with single channel connections on div 1. A single cell adhered to the agarose next to one well (arrowhead). (b) The same culture on div 4. Cells developed extensive neurite outgrowth. Numerous neurites follow the closed micro channels. The soma of the neuron in the lower left corner (arrow) moved during the culture period. The cell on the agarose surface (arrowhead) was still present but looked condensed and no longer viable. (c) The same culture after fixation on div 4 and staining with α-tubulin antiserum. Immunostaining demonstrates the extent of neuritic branching and confirms growth restriction to the agarose-free areas. Please note that several neurites have reached adjacent wells by growing through the microchannels. The cell located on the agarose layer (arrowhead in a) and b)) was lost during staining procedure indicating weak adhesion. Scale bar: 100 μm.

div 1 and 4. They were cultured in a standard culture dish surface without surface coating. The neurons that were manually placed into the wells adhered to this substrate (Figure 6a) and developed neurites within the following days (Figure 6b). Neurites followed the channels into adjacent wells without any need for other promoting guiding cues but the physical cue of the channel and the chemical nonadhesive property of the agarose itself. Thereby, the dimensions of the micro channels seemed to play a critical role. Preliminary experiments with different channel dimensions showed that smaller channels of width and height of 5−10 μm yielded better guidance than larger ones of 30 μm width and height.

In general, the cells did not bridge the well edges of the agarose layer, thus branching was confined to the wells and channels. Staining showed this precise confinement of neurite branching even better (Figure 6c). Comparable results could be achieved with *Locusta* neurons in agarose well arrangements with single or triple channel connections (Figure 7).

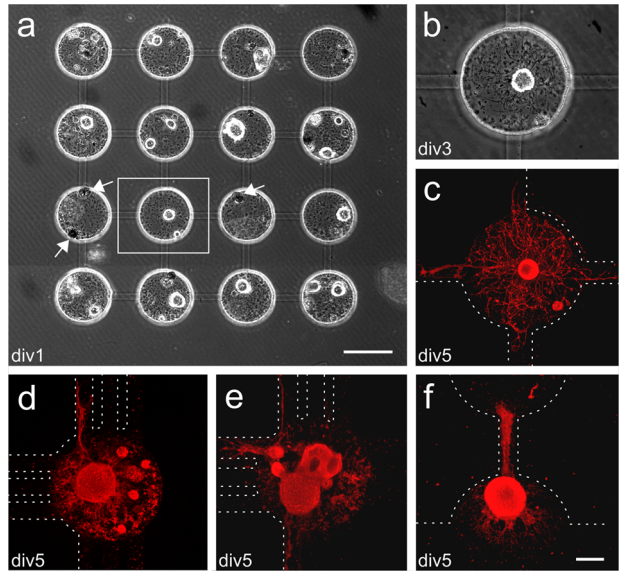

Figure 7. Neural growth within agarose wells (*Locusta*). (a) *Locusta* neurons positioned in wells of a 16-well arrangement with one channel connection on div 1. Almost every well is occupied by 1−3 vital neurons. Arrows indicate dead neurons. (b, c) One of the 16 wells (indicated by the rectangle in a)) at higher magnification on different days of culture. (b) Neuron on div 3 shows viability as it began to develop multipolar neuritic branching. (c) Neuron morphology on div 5 after horseradish-peroxidase (HRP)-immunostaining which visualizes the whole neuritic extent. (d−f) HRP-immunostained neurons on div 5 as further examples which illustrate that neurite outgrowth was confined to the wells and channels. Dashed lines indicate the borders of the agarose templates. Scale bars: (a) 100 μm, (b−f) 40 μm.

Cell positioning could also be achieved by increasing the cell density per application and dispersing the cells randomly on the agarose slice, where generally several neurons occupied one well (Figure 8). Cell adherence and neuritic growth confined to the wells was also observed within the later days of culture on these MEA surfaces (Figure 9). Pond snail neurons in some cases adhered to the surrounding agarose surface first, but the number of such cells generally rapidly declined until div 4 (Figure 9a).

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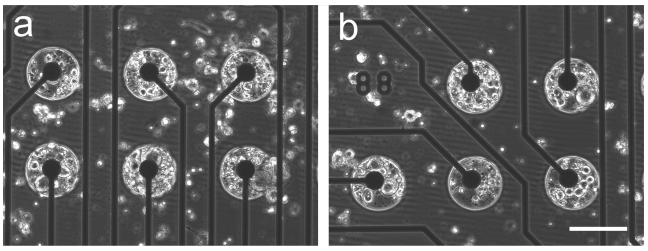

Figure 8. Neuron positioning in wells of an agarose slice on a MEA. Pictures (a) and (b) show two examples of *Lymnaea* neurons on div 1 applied by chance to the agarose slice. Neurons became positioned to the wells and adhered to the MEA surface. Most of the few neurons that ended up on the agarose layer did not manage to adhere to it, but swam in the medium. Scale bar: 100 μm.

4. DISCUSSION

An innovative technique for substrate patterning with agarose is presented. Exact molding from microstructured templates could be achieved reliably, and production of thin slices with a vibratome provided stable agarose substrate modifications of consistent height and reproducible quality. Agarose slices showed long-term attachment to several different substrates without any additional treatment. The well and channel pattern succeeded to guide neuronal growth. Neurons adhered to the agarose free areas, whereby their somata were confined to the wells, while neurites were free to also grow into the channels. Thereby, it is assumed that the physical impulse given by the narrow path impressed the neurites to grow aligned.

4.1. Methodology. In the literature, different but related methods of substrate production using agarose or other hydrogel or elastomer materials were described previously.[10,31,32,43,44] They allow for the production of several kinds of microwells, but not that of microchannels for a guided pathfinding of neurites. Some of the techniques show limitations in adjusting and achieving appropriate well depths.[31,32] The combination of microwells and channels in an agarose substrate for positioning and neurite guiding was shown by different studies.[9,15,45] These authors used photothermal etching processes or similar laser technology that demand relatively high technical effort for creating the channels. In contrast, our approach using in parallel processed templates with well-defined microstructures on a silicon wafer, is able to produce wells and closed microchannel within one molding step. Once the wafer is obtained, molds and slices can be prepared by simply casting and molding. Furthermore, the slice production technique by using a vibratome offers flexibility as the adjustment of any desired slice thickness can be achieved easily and fast on demand.

Deviations in slice thickness are most likely due to a swelling of the hydrogel during long-term incubation in medium. Until now, the production of accurate layer thicknesses was most likely only possible with spin-coating technologies.[46] Otherwise, layer production of defined height may be challenging. The method presented here for the production of slices via vibratome shows a new approach to achieve reproducible and almost constant layer thickness.

In our experiments, adherence of agarose slices on different substrates can be maintained for 3 weeks or longer if optimal experimental conditions are provided. This time span is sufficient for locust and snail neuron culture. Network development reaches its maximum within the first week. Furthermore, agarose slices, although showing good attachment

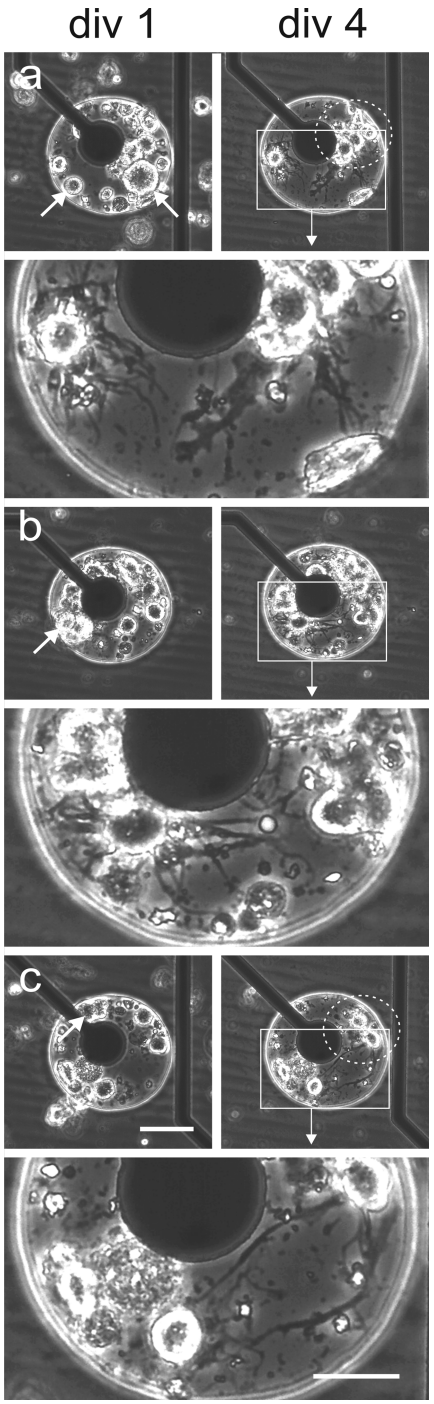

Figure 9. Network development within wells on MEA device. Pictures (a–c) show *Lymnaea* neurons within different wells of the same MEA on div 1 and div 4 for comparison of development. Within 4 days in culture, neurons developed neurites that grew within the borders of the wells (for a detailed view on neurites see corresponding insets indicated by rectangles in the div 4 row of (a), (b) and (c)). Cells that did not achieve proper adhesion on div 1 detached until div 4 and disappeared (white arrows). Several neurons showed mobility and indicated a tendency to assemble in clusters until div 4 (dashed line circles). The number of cells outside the wells on the agarose layer declined between div 1 and div 4. Scale bars: (c) 20 μm, inset 40 μm.

to MEAs or different reusable devices, can be removed after completion of an experiment and replaced by new slices which facilitates cleaning procedures. This point is not necessarily

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given if guiding modifications are irreversibly fixed to the devices.[18,43,44]

4.2. Guiding by Physical Cues. Finding optimal conditions for constructing networks of neurons is a critical point. Guiding by chemical cues alone, performed by microcontact printing or patterned laser ablation of adhesive coatings for example, is often only applicable to a limited number of cell types. In addition, the mentioned techniques do not allow for a differentiation between soma position and neurite path in general.[47] Then somata and neurite branching are randomly distributed over the printed grid. Furthermore, cells in culture often show extensive motility due to tension originating from growing neurites.[48,49] For these reasons, pattern quality of networks constructed by chemical cues alone can be limited,[12,47,50,51] especially over longer periods of time.[22] Previous studies already revealed that chemicals might play a more minor role in cell extension guiding compared to raised topographical modifications.[17] Other studies showed that the direction of the outgrowth of cell extensions in culture is strongly influenced by the surface topography, as in dependence of the type cells tend to follow certain surface profiles.[52] Hence, cell pattern control studies focus more and more on the physical confinement of cells in culture. However, as many cell types, especially from vertebrates, need chemical substrate modifications to adhere at all, one has to bear in mind that in dependence of the cell type a sole physical confinement probably does not suffice either.[9] If cell adherence to the substrate is warranted, the agarose slices presented here with open wells and closed channels provide a good guiding quality by physical impulses applicable for various cell types. The closed channels prevent somata from occupying the neurite path, the low height of the channels prevents somata from migrating into them, and the channel dimensions seem to provide a physical impulse to the neurites to grow aligned. The raised wells act as a physical barrier which immobilize somata and prevent neurites from overcoming it.

4.3. Cell Culture in Wells. The observation of negative influences on long-term survival of cells in wells of 120 μm height in comparison to the 50 μm high wells, might be due to an impediment of medium exchange and cell waste product accumulation in the deep cavities without medium flow. Insufficient nutrient supply and waste disposal, therefore, might result in a limited cell survival. As a result, dependent from cell type, the minimum required thickness of agarose hydrogel slices that ensures stable neuron positioning should be used. Reliable stable agarose slices down to a height of under 30 μm are possible to produce which should be sufficient for vertebrate neurons for example. But as a reliable immobilization of locust and pond snail neurons could only be achieved with 50 μm high wells and up as their neurons have large diameters it was chosen to work with thicker slices.

For achieving isolated cell clusters with low cell number but less defined network structure in the wells without channels presented here, up to 7 invertebrate neurons were collected per well, as well diameter used here did not allow for a higher cell density. If requested, this limitation can be overcome by adjusting the pin diameter of the silicon templates, which would result in greater wells. Besides, as vertebrate neurons have lower diameters, here cell density could be raised even in smaller wells.

4.4. Agarose as Patterning Material. Invertebrate neurons, in comparison to vertebrate neurons, are comparatively unselective concerning the substrate on which they grow. This unpretentiousness impedes growth cone guiding by adhesive cues as often used with vertebrate neurons. Indeed, growth guiding of *Lymnaea* neurons by chemical cues was managed previously by a substrate patterned with poly-L-lysine and brain-derived conditioning factors.[14] In our experience, however, *Lymnaea* neurons do not need any chemical coating to achieve strong adhesion to various culture substrates such as polystyrene, glass, or MEA surfaces. For this reason, chemical patterns here do not influence neurite growth as much as it is desired. The same is true for *Locusta* neurons. Therefore, we focused on a structured and nonadhesive material to provide physical guiding in combination with repulsive chemical cues, rather than adhesive ones. Agarose is an established material, at least for vertebrate cell guiding strategies,[15,19,31,53] as it is nonadhesive for most of the cell types tested, but to our knowledge agarose was not used before to guide invertebrate neurons. For example, polydimethylsiloxane (PDMS) and similar silicones are prominent alternatives to agarose in substrate patterning technologies and are as well widely used for vertebrate cell guiding,[45,54,55] especially in combination with microfluidics.[56−60] PDMS was already shown to be an almost nonadhesive material for locust neurons in microfluidic devices.[61] For *Helix* neurons in culture it was shown that adhesion is not impeded on PDMS.[46,62] *Lymnaea* neurons also did not show any restricted growth on PDMS compared to control groups growing on glass or polystyrene (data not shown). And adherence of *Locusta* neurons is only strongly restricted as about 20% of cells adhered to PDMS in comparison to the control group cultured on polystyrene.[61] Because of this, PDMS turned out to be unsuitable for our purposes. Other synthetic polymers that act as nonadhesive surfaces are polyethylene glycol (PEG),[63−65] polyacrylamide,[53,66] or pluronics[53,67] for example. Only PEG provides a nonadhesive surface modification for insect neurons,[64] but due to a difficult production process and poor reproducibility, this material appears unsuitable for routine applications.

Agarose, in contrast, has many practical advantages: It offers easy conversion from gel to liquid, making gel production and curing manageable within minutes. Sterility can easily be achieved by autoclaving. Agarose is a natural, noncytotoxic, and relatively cheap material. Finally it is transparent, which allows for the direct observation of cell behavior even in closed channels.

CONCLUSION

A new process based on photolithography and micromolding techniques is presented for the fabrication of structured agarose slices to create geometrically defined topologies for the controlled development of neuronal networks in culture. Further substrate modifications are not necessary. The slices were tested on conventional cell culture substrates (glass, polystyrene), but also on different types of multielectrode arrays (MEAs). The procedure provides high flexibility as many parameters are easy to adapt to specific demands. Experiments with locust and pond snail neuron cultures demonstrated the great potential of the agarose slices for positioning and immobilizing cells. In addition, micro channels integrated into the slices are sufficient to guide directional growth of neurites. The combination of a chemically nonattractive material with a suitable topology most certainly allows the investigation of a large range of cell types and various analysis methods.

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Author Contributions

§K.K. and J.R. contributed equally. The manuscript was written by K.K. Authors K.K., J.R., P.B., and K.B.G. were involved in the development of the conceptual design of the production technique and its application. The experimental part in terms of technique development, cell culture, and their examinations was done by K.K. and J.R. A.E.H. and U.S. provided production of the silicon wafer for pattern molding. The study was supervised by K.B.G. All authors have given approval to the final version of the manuscript.

Notes

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