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Patterned networks of mouse hippocampal neurons on peptide-coated gold surfaces

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Abstract

Patterned networks of hippocampal neurons were generated on peptide-coated gold substrates prepared by microscope projection photolithography and microcontact printing. A 19 amino acid peptide fragment of laminin A (PA22-2) that includes the IKVAV cell adhesion domain was used to direct patterns of cell adhesion in primary culture. Microscale grid patterns of peptide were deposited on gold-coated glass cover slips by soft lithography using "stamps" fashioned from polydimethylsiloxane. Strong coordination bonding between gold atoms on the surface and the sulfur atoms of the N-terminal cysteine residues supported stable adhesion of the peptide, which was confirmed by immunofluorescence using anti-IKVAV antiserum. Dispersed hippocampal cells isolated from neonatal mouse pups were grown on peptide-patterned gold substrates for 7 days. Neurons preferentially adhered to peptide-coated regions of the gold surface and restricted their processes to the peptide patterns. Whole cell recordings of neurons grown in patterned arrays revealed an average membrane potential of −50 mV, as well as the presence of voltage-gated ion conductances. Peptide-modified gold surfaces serve as convenient and effective substrates for growing ordered neural networks that are compatible with existing multi-electrode array recording technology.

Keywords: Microcontact printing; Hippocampus; Neuron; Neural network; PA22-2; Gold

1. Introduction

Neurons grown in cell culture make synaptic connections to form interactive networks. The random nature of network organization in culture complicates the investigation of synaptic interactions. Thus, a strategy for creating cultured neural networks with defined composition and connectivity is needed. The availability of ordered neural networks would facilitate the investigation of patterns of synaptic interaction, the control of synapse formation, and cell signaling processes using highly parallel electrophysiological and optical recording techniques.

A useful approach for controlling connectivity between cultured neurons is the creation of microscale patterns of cell adhesion molecules (CAM) on the culture substrate by microcontact printing [1–9]. Geometric patterns cast in polydimethylsiloxane (PDMS) serve as "stamps" for the transfer of CAMs, or other affinity reagents, to properly prepared substrates [10–12]. The Offenhausser group reported the creation of an ordered neuronal culture on a Cartesian grid of laminin A [1,4], which featured an array of $14 \times 14\,\mu m$ nodes connected by $6\,\mu m$ tracks. Isolated neurons cultured on patterned CAM grids assemble into ordered arrays with cell bodies confined to nodal regions and cell processes extending along the internodal tracts. Such a design is nicely compatible with current multi-electrode array

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recording technology, and would also be amenable to optical recording methods.

Previous patterning strategies have focused on neural cell growth on glass or silicon surfaces. Various ω-terminated trialkoxysilanes have been employed to functionalize glass surfaces and to attach extracellular matrix proteins (EMPs) or protein fragments [4,7,8]. Other investigators have patterned EMPs directly onto surfaces via physisorption [1,2,9]. The use of gold-coated glass as a substrate for the generation of patterned neural cultures is a particularly attractive approach because (1) peptides with N- or C-terminal cysteine residues bind strongly to gold without the need for additional chemical modification [10], and (2) the conductive nature of the metal facilitates electrical coupling of individual neurons to electrophysiological recording and stimulating apparatus. In the current study, we describe a procedure for the creation of ordered neural networks on stable patterns of the cell adhesive peptide, PA22-2 [13] deposited by microcontact printing directly onto an unmodified gold substrate.

2. Materials and methods

2.1. Microscope projection photolithography

Molds for the production of PDMS stamps were produced as described by Love and coworkers [14]. A photomask (11 mm in diameter) was created with a CAD drawing tool (TurboCAD Professional v8; San Rafael, CA) and printed on transparency film at a resolution of 5080 dpi (Pageworks; Cambridge, MA). The pattern on the photomask was scaled 10 times larger than the desired dimensions of the stamp (6 μm line widths spaced 50 μm apart, with nodes 14×14 μm wide [1,4]). To transfer the reduced image to a substrate, the photomask was placed at the field stop of an Olympus BX51WI microscope. A 75 W xenon lamp was used to project broad-spectrum light through the pattern, which was focused with an Olympus $40\times$ water immersion objective onto a silicon wafer coated with photoresist. An infrared-reflecting hot mirror (NT43-842; Edmund Industrial Optics; Barrington, NJ) was used to protect the photomask from the heat of the lamp.

To prepare silicon wafers for the deposition of photoresist, Si(1 0 0) wafers (Silicon Sense; Nashau, NH) were sonicated in acetone and methanol and dried at 180°C. Positive photoresist (Shipley Microposit 1813; Shipley Company; Marlborough, MA) was spin-coated onto the wafer at 4000 rpm for 40 s to a thickness of approximately 1.3 μm with a Laurel WS-400A-6NPP/Lite spin-coater (Laurel Technologies Corp.; Johnstown, PA). The coated wafers were baked at 105°C for 4 min. Photoresist-coated silicon was protected from light during preparation. The coated wafers were exposed to the xenon lamp for 10 s and developed in Microposit MF-320 (Shipley Company; Marlborough, MA) for 1 min to create a pattern approximately 0.5 mm in diameter.

2.2. Fabrication of stamps

PDMS stamps were fabricated from the photolithographic patterns using Sylgard 184 Silicone Elastomer (Dow Corning Corporation; Midland, MI). The polymer was poured over a 3×3 array of patterns and allowed to cure for 24 h at 65°C (Fig. 1). The resulting stamps were cut out of the polymer, washed in ethanol, and stored in distilled water.

2.3. Glass preparation and gold deposition

Glass cover slips (Fischer Scientific; Pittsburgh, PA) were soaked in a solution of three parts 30% hydrogen peroxide and seven parts sulfuric acid for 10 min (*Caution: this mixture, known as "piranha solution",*

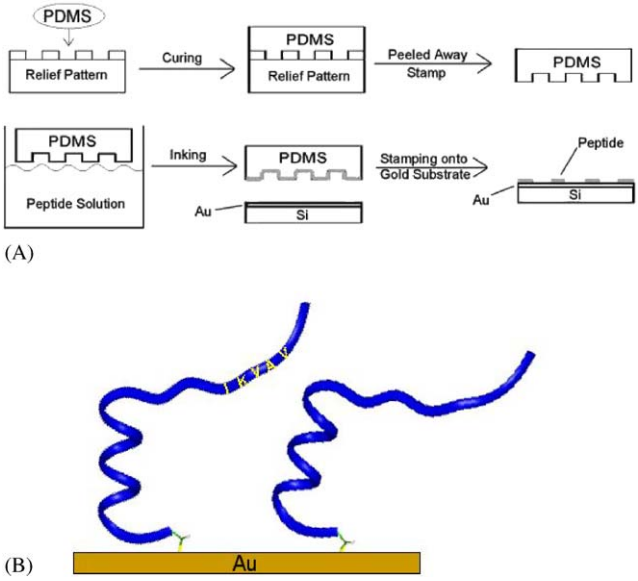

Fig. 1. Microcontact printing of PA22-2. (A) Stamp preparation: an image of the desired pattern is "burned" onto photoresist by microscope photolithography (see Methods) [14]. This relief surface serves as a rigid mold for the production of PDMS stamps. The stamp is "inked" with a solution of peptide, and dried under a stream of nitrogen, and the peptide is transferred to the unmodified gold surface. (B) Hypothetical conformation of PA22-2 peptide on gold. Strong coordination bonding between the sulfur atoms of the N-terminal cysteine residues and the gold lattice secures the peptide to the surface. The alpha helical conformation of the N-terminal half of the peptide facilitates display of the IKVAV cell adhesion domain. Conformational analysis of the peptide was conducted using the AGADIR Program (http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start.html). The image was calculated and rendered using insightII molecular visualization software (Accelrys, Inc.; San Diego, CA).

reacts violently with organic matter and should be handled with extreme care!). Treated cover slips were rinsed thoroughly with distilled water, and boiled in a solution of one part 30% hydrogen peroxide, one part ammonium hydroxide, and five parts distilled water for 30 min at 80°C. The cleaned cover slips were rinsed again with distilled water, and refluxed for 10 min at 90°C in a solution of 5 g of 3-mercaptopropyl-trimethoxysilane in 30 ml of 25% isopropyl alcohol. The functionalized cover slips were rinsed again with distilled water and dried under a stream of nitrogen. A thin layer of gold (150 Å) was deposited onto the functionalized cover slips by vacuum evaporation. The gold layer was sufficiently transparent to permit observation of cell morphology by phase contrast microscopy.

2.4. Microcontact printing

Prior to stamping, gold-coated cover slips and PDMS stamps were immersed in 70% ethanol for 20 min, washed with distilled water, and dried under a stream of filtered (0.2 μm) ultrapure nitrogen. Stamps were "inked" with a solution of 5 μM PA22-2 peptide (NH_2–Cys–Ser–Arg–Ala–Arg–Lys–Gln–Ala–Ala–Ser–Ile–Lys–Val–Ala–Val–Ser–Ala–Asp–Arg–CO_2H; Sigma Chemical Co.; St. Louis, MO) in sodium phosphate buffer (PB) (50 mM, pH 7.0) for 1 min and dried in a stream of filtered, ultrapure nitrogen. Stamps were in contact with the gold surface for 1 min. Peptide-coated gold surfaces were rinsed with distilled water, and stored dry at 4°C until use (up to 2 weeks).

2.5. Atomic force microscopy

Tapping mode AFM images were collected under ambient atmospheric conditions with a Multimode Nanoscope IIIa (Digital Instruments) using an Ultrasharp NSC12 Si cantilever (Silicon-MDT Ltd.). Low-resolution scans showed systematic differences in sample height across the surface, which was interpreted to reflect the presence of alternating coated and uncoated regions (vide infra).

2.6. Cell culture

Neonatal C57BL/6 mice (*Mus musculus*) were obtained from pregnant dams raised in our animal facility. The hippocampus was dissected from the brains of newborn (1–7 days old) male and female mice. Dispersed cells were prepared for culture using a commercial kit (Papain Dissociation System, Worthington Biochemical Corporation; Lakewood, NJ). Enzyme activity was quenched by the addition of 2% neonatal calf serum. Cells were cultured in Neurobasal-A growth medium without phenol red, containing 2% B-27 growth supplement, 0.5 mM L-glutamine, 50 units/ml penicillin, and 50 mg/ml streptomycin (Invitrogen Corporation; Carlsbad, CA). Aliquots (100 μl) of the cell suspension (2×10^4 cells/ml) were plated onto the patterned substrates. After 10 min, 2 ml of fresh cell culture medium was added, and the cultures were placed in an incubator at 37°C under a humidified atmosphere of 5% CO_2 in air. The initial culture medium was replaced after 1 h; subsequently, the medium was changed every 4 days. After 1 h, 1, 3, and 7 days in culture, some cover slips were removed and fixed in either pure methanol for 20 min at −20°C, or a solution of 4% paraformaldehyde in sodium (PB; 0.1 M, pH 7.4, 0.01% Thimerosal) for 10 min. All cover slips prepared in this manner were stored in PB at 4°C until processed for immunocytochemistry.

2.7. Characterization of cultured cells

Patterned cell cultures were processed for immunofluorescence using standard techniques. Briefly, fixed cultures were incubated for 1 h at room temperature in each of the following solutions: (1) blocking solution comprised of 1.5% goat serum (NGS) (Sigma) in PB with 0.3% Triton X-100 (PBT), (2) primary antiserum (1:200 rabbit anti-IKVAV; kindly provided by Dr Kleinman, NIDR-NIH); or 1:1000 rabbit anti-MAP-2 (Chemicon International Inc.; Temecula, CA), and (3) secondary antibody (2 mg/ml; Alexa 488-conjugated goat anti-rabbit IgG or Alexa 568-conjugated goat anti-rabbit IgG) (Molecular Probes). For triple label immunofluorescence, methanol-fixed cultures were incubated first with rabbit anti-MAP-2 (1:1000) followed by goat anti-rabbit Alexa 568 conjugate (1:2000), and subsequently with chicken anti-glial fibrillary acidic protein (anti-GFAP; 1:1000) (Chemicon International Inc.; Temecula, CA) followed by goat anti-chicken Alexa 488 conjugate (1:2000). Cell nuclei were stained with DAPI nuclear stain (Molecular Probes; Eugene, OR). Fluorescent cells were observed using a Zeiss 310 confocal laser scanning microscope (Carl Zeiss; Oberkochen, Germany). Alexa dyes were excited with the 488 and 568 nm lines of an argon–krypton laser (Melles Griot; Carlsbad, CA). DAPI imaging was achieved using an argon ion laser tuned to 364 nm (Coherent; Santa Clara, CA). Phase contrast images of cultured arrays were obtained with an Olympus IMT-2 inverted microscope and photographed using an ORCA II cooled CCD camera (Hamamatsu; Hamamatsu City, Japan).

2.8. Electrophysiological recording

The electrophysiological properties of cultured neurons were evaluated using the whole cell patch-clamp technique as described previously [15]. Recordings were obtained after 4 and 7 days in culture. Borosilicate glass

pipettes were filled with an electrolyte solution (5 mM EGTA, 10 mM HEPES, 1 mM $MgCl_2$, 130 mM potassium gluconate, 1 mM NaCl, 1 mM CaCl, 2 mM K_2ATP and 10 μM 5- (and 6-) tetramethylrhodamin biocytin (biocytin TMR) dye (Molecular Probes; Eugene, OR) and positioned using a Sutter MP-285 Motorized Micromanipulator (Sutter Instrument Company; Novato, CA). The extracellular medium consisted of 122 mM NaCl, 3.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl \cdot H_2O$, and 10 mM dextrose. Data were acquired using an Axon Instruments Axopatch 1D amplifier (Axon Instruments; Burlingame, CA) and a Mac OS-X computer running custom data acquisition software (DAM; kindly provided by Dr. Costa Colbert, University of Houston). Neurons were held at a potential of −70 mV before applying command potentials between −100 and +60 mV in 20 mV increments. Voltage-gated inward and outward currents were measured.

3. Results

Photoresist relief patterns with feature heights of 1.2–1.3 μm were produced using microscope-projection photolithography [14]. Each 10-s exposure to light afforded a patterned area measuring approximately 0.5 mm in diameter. By making multiple exposures on the same piece of silicon, we were able to create an arbitrarily large array of patterns (e.g., 22 or more individual patterns on a single silicon wafer). As described above, complementary PDMS stamps for microcontact printing were then readily fabricated from these relief molds.

Inking of the stamps with PA22-2 peptide followed by microcontact printing directly onto gold substrates led to well-ordered patterns of the peptide on gold. Epifluorescence observations of PA22-2 peptide immunostained with anti-IKVAV and Alexa-488 conjugated IgG revealed that the peptide covered a well-defined area on the substrate with clear boundaries separating bare regions from those coated with peptide (Fig. 2). Linewidths varied from 6 to 8 μm, and the nodes occupied approximately 200 μm^2. Nodes were separated diagonally by ~60 μm. The areas between lines were largely devoid of peptide, except on rare occasions (presumably due to excess pressure applied during stamping).

Analysis of peptide patterns by AFM revealed an average SAM height of approximately 1.5 nm above the surface of the gold, with an RMS roughness of ~1 nm (Fig. 2). The observed peptide patterns were consistent with those expected from the patterns on the stamp. Detailed AFM measurements on peptide-coated regions showed neither domain formation nor long-range order, which is consistent with a SAM that lacks conformational order (i.e., loose packed).

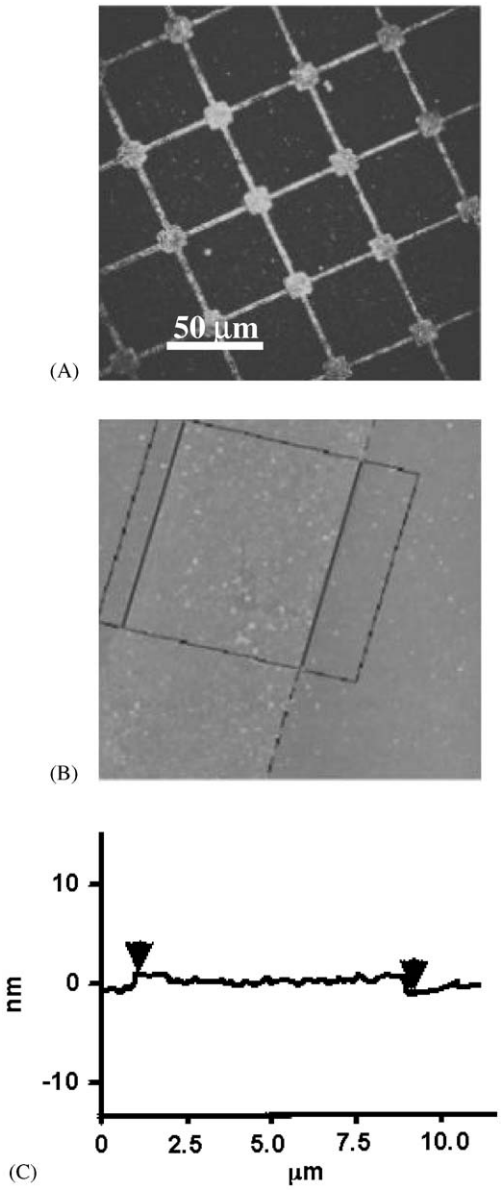

Fig. 2. AFM analysis of PA22-2-coated gold surfaces. (A) Fluorescence image of PA22-2 deposited onto gold-coated glass. White bar = 50 μm. (B) AFM image of PA22-2-coated tract. (C) Plot of the surface topography across a PA22-2-coated tract. The peptide layer was approximately 1.5 nm thick, consistent with SAM formation.

Mouse hippocampal neurons were plated onto the PA22-2 stamped substrates to assess the ability of neurons to form patterns on gold. On cover slips fixed after 1 h of incubation, an apparently random pattern of adherent cells without processes was observed. After 24 h in culture, regions of patterned cell growth were apparent in which adjacent neurons extended processes at right angles to one another. Observation of cover slips processed for IKVAV immunofluorescence after 1 day in culture revealed that cells were in contact with the PA22-2 peptide patterns (Fig. 3). Cell bodies tended to rest on the 14 μm nodes, while neurites extended along

the thinner peptide lines. Few, if any, cells were observed in the areas of unmodified gold between peptide tracts.

The cellular composition of 4- and 7-day hippocampal cultures was determined using immunocytochemical markers for astrocytes (GFAP) and neurons (MAP-2). After 4 days in culture, both GFAP and MAP-2 immunoreactive cells were observed on peptide-free regions of gold substrates (Fig. 4A). However, few GFAP-immunoreactive cells were observed on PA22-2 grids. Some patterned cell networks were composed entirely of MAP-2 immunoreactive cells (Fig. 4B).

The relative affinity of cultured neurons for PA22-2 peptide versus underivatized gold was assessed. A flat, unpatterned PDMS stamp was inked with PA22-2 peptide. One-half of a gold cover slip was stamped with the peptide, while the other side was left unmodified (Fig. 5). Cultured neurons were plated in 500 μl of cell medium to ensure that surfaces were uniformly covered with cell suspension. After 7 days in culture, the distribution of cultured cells on both surfaces was assessed by phase contrast microscopy. Neurons deposited on uncoated gold, in the absence of peptide, were observed to attach, survive for 7 days, and extend processes along the gold surface. However, when cells were deposited in close proximity to the PA22-2, a clear preference of the cells for the peptide was apparent (Fig. 5). A clear line of cells defined the peptide/gold boundary, with fewer cells present on the unmodified gold adjacent to the peptide, suggesting that some cells migrated from the unmodified gold surface to achieve contact with the peptide.

After 7 days in culture, cells with neuronal morphology growing in association with PA22-2 grids were subjected to whole cell patch recording to assess their electrophysiological characteristics (Fig. 6). Resting membrane potentials of cultured neurons ($n = 6$) were between −45 and −55 mV, values that are reasonable for mammalian neurons. Depolarization of patched neurons evoked rapid, transient, inward currents followed by delayed outward currents.

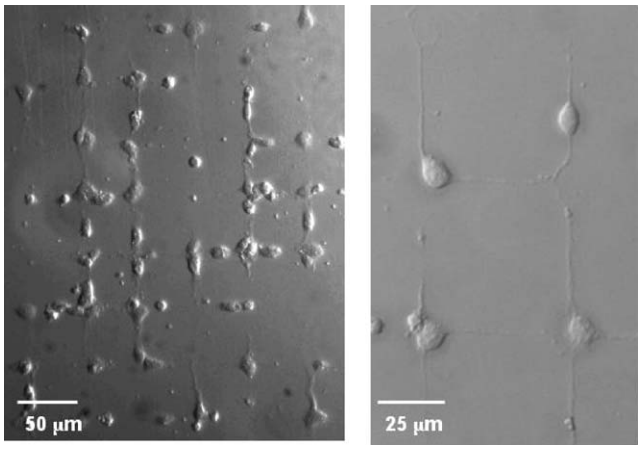

Fig. 3. Differential interference contrast images of patterned cultures of hippocampal neurons after 4 days in culture.

4. Discussion

Microcontact printing of CAMs and other affinity reagents has proven useful as a means of achieving ordered patterns of cell growth in vitro. In the current report, we demonstrate that stable patterns of the cell adhesive peptide, PA22-2, can be formed on unmodified gold substrates, and that this surface supports the survival of mouse hippocampal neurons for at least 7 days in culture. Immunocytochemical observation of

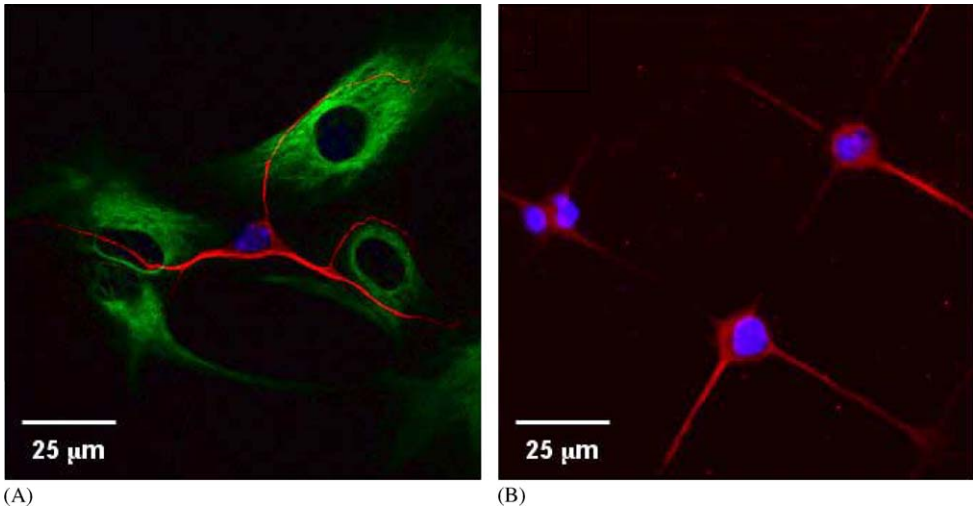

Fig. 4. Most of the hippocampal cells growing on peptide patterns are neurons. Confocal micrographs of hippocampal cells after 4 days in culture on (A) poly-D-lysine-coated glass cover slips, or (B) patterned PA22-2 on gold. Both preparations were cultured under identical conditions, fixed and immunostained as described above. Green signal indicates the presence of GFAP immunoreactivity, while MAP-2 immunoreactivity is shown in red. Cell nuclei (blue) are stained with DAPI.

these patterned cultures indicated that most of the adherent cells are neurons with mature processes that extend along the peptide-coated tracts for hundreds of microns. Whole cell recordings of hippocampal neurons after 7 days of culture on PA22-2 patterns revealed transmembrane potentials and voltage-gated ionic conductances that are characteristic of mature neurons. Thus, it is reasonable to speculate that neurons cultured in this way establish active networks that may display many of the emergent properties of in situ networks.

Microscope projection photolithography [14] was found to be an inexpensive and useful method for the creation of PDMS stamps in order to successfully execute microcontact printing on gold. By drawing the photomask using commercial CAD software, printing on transparency film, and conducting the photolithography on the same microscope used for imaging of cells, this technique proved to be an inexpensive alternative to outsourcing small lithography jobs, especially when a variety of different photomasks were required.

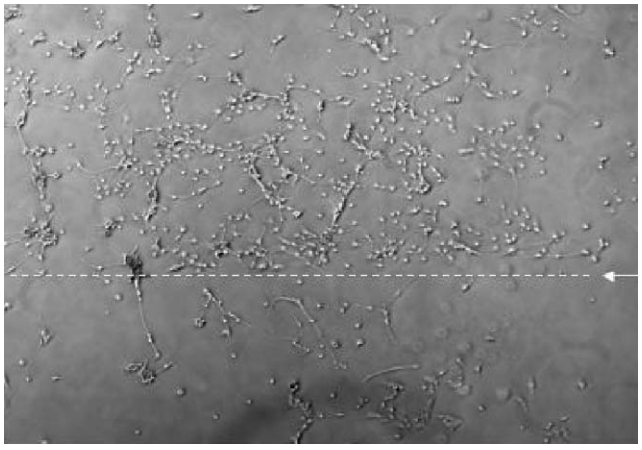

Fig. 5. Cultured hippocampal cells prefer peptide-coated gold. Phase contrast image of hippocampal cells grown on a gold surface, half of which is coated with PA22-2. The top half of the image shows the peptide-coated region, while the bottom half is uncoated gold. The dotted line indicates the approximate border between coated and uncoated regions. After 4 days in culture, more of the surviving cells are attached to the peptide-coated regions of the gold surface.

Microprinting of unmodified gold surfaces with PA22-2 led to stable attachment of the peptide, presumably via the N-terminal cysteine residues. AFM analysis indicated that microstamped PA22-2 peptide produced a non-uniform monolayer with an average height of approximately 1.5 nm. The observed variation in the height of the peptide features is consistent with the formation of a loosely packed monolayer. SAMs derived from normal alkanethiols are typically more densely packed and conformationally ordered. However, the relatively loose packing in the PA22-2 SAMs is desirable, as this should allow the IKVAV binding domain, which begins five residues from the unattached end of the PA22-2 peptide, to lie exposed for cell adhesion. In contrast, access to functional groups along the axis of the molecular adsorbate in normal SAMs is often severely restricted.

Mouse hippocampal neurons were observed to form patterned networks on unmodified gold substrates printed with PA22-2 peptide. This observation is consistent with previous reports employing patterns of PA22-2 on functionalized glass surfaces [4]. Regular patterns of neurons self-organized on the peptide by 24 h in culture and survived on this substrate for at least 7 days. Although an ethylene glycol SAM [11,16] is often used to discourage cell adhesion to bare gold surfaces, very few neurons were observed in contact with bare

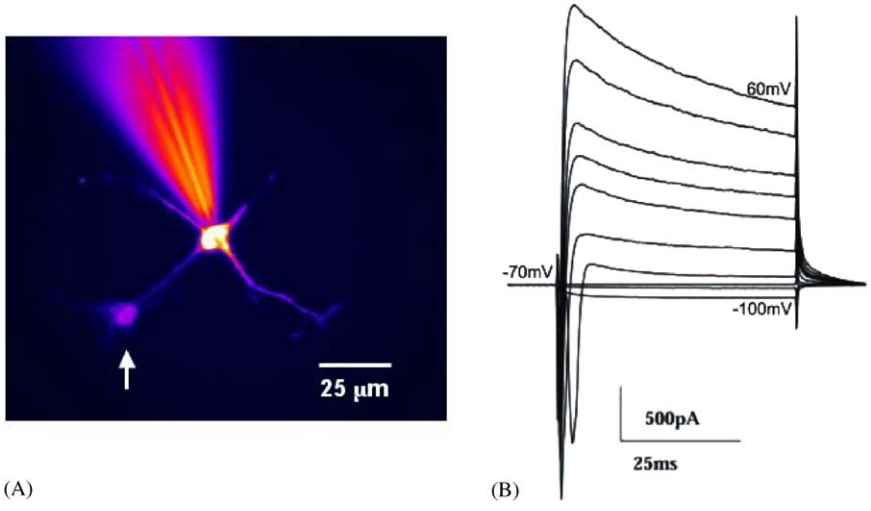

Fig. 6. Hippocampal neurons growing on PA22-2 patterns display voltage-gated ionic conductances. (A) Pseudo-color image of a patched neuron filled with biocytin-TMR. Note the restriction of labeled processes to the peptide-coated grid. In this case, a second labeled cell was also filled with the dye (arrow), suggesting the presence of gap junctions between these two cells. (B) Voltage-dependent currents evoked in a cultured hippocampal neuron. Depolarizing pulses induce transient inward currents followed by sustained outward currents, which is characteristic of mature neurons.

gold areas within the peptide-coated grids, even without such an anti-adhesive barrier. Furthermore, when cells were deposited at the edge of an unpatterned peptide-coated region (Fig. 5), the boundary between peptide-coated and bare gold regions show a marked difference in cell density, with most neurons appearing on peptide-coated areas. It is unclear from the present study whether cells plated onto bare gold failed to adhere to the surface, or migrated onto peptide-functionalized areas. It is also possible that different cellular phenotypes vary in the preference for the peptide. Nonetheless, some cells survive in culture for over a week on the bare gold surface, indicating that gold is non-cytotoxic to hippocampal neurons and is a suitable surface for the cell culture. Collectively, these observations suggest that it should be possible to grow specifically patterned neural cells and networks directly on peptide-coated gold surfaces without the need for codepositing ethylene glycol moieties.

Under the culture conditions employed in the current study, most of the cells observed in association with PA22-2 patterns after 4 days in culture were neurons. These cells displayed neuronal morphology and were immunoreactive with antiserum that recognizes the neuron-specific protein, MAP2. In agreement with previous reports [4,9] electrophysiological observations confirmed that these cells exhibited membrane potentials and voltage-gated ionic conductances that are characteristic of mature neurons. Thus, with appropriate surface modifications, it may be possible to construct defined neural networks in vitro.

5. Conclusions

Dispersed mouse hippocampal cells form patterned networks of active neurons on gold substrates printed with PA22-2 peptide. These cells remain viable for at least 7 days in culture and may form functional networks with properties similar to those of in situ networks. Future studies will explore the use of optical methods for observing spontaneous and evoked responses of similarly patterned neural networks. This approach could provide a useful model for the investigation of the neurophysiological and biochemical processes that determine the formation and behavior of defined neural networks in vitro.

Acknowledgements

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