Patterning to enhance activity of cultured neuronal networks

Y. Nam, J. Chang, D. Khatami, G.J. Brewer and B.C. Wheeler

Abstract: Embryonic rat hippocampal neurons were cultured in order to gain insights into how small networks of neurons interact. The principal observations are the electrical activities recorded with the electrode arrays, primarily action potentials both spontaneous and evoked. Several lithographic techniques were developed for controlling with micrometer precision the patterns of surface molecules in order to control neuronal attachment and growth. Cytophilic polylysine against protein repellent and hence cytophobic polyethylene glycol were used. By combining the cellular lithography with the microelectrode arrays it was possible to guide neurons preferentially to electrodes and to begin to investigate the question as to whether the geometric pattern of a neuronal network influences the patterns of its neuroelectric activity. It is clear that the techniques are adequate to ensure contact of neurons to electrodes but not to ensure the recording of signals, even when neurons lie directly on top of electrodes. The maturation of neuroelectric activity depends on the growth of glia within the culture, such that spontaneous activity appears to become robust when the number of glia is roughly the same as the number of neurons.

1 Introduction

The last decade has seen rapidly growing interest in the use of self-assembled monolayers to control growth and expression of cultured cells, of micro lithography to control the geometrical patterns in which these cells grow, of culturing neurons on top of microelectrode arrays (MEAs) for stimulation and recording, and of combining these technologies to create defined circuits of neurons with controllable connectivity and function. This report targets a critical component of the combination; the ability to ensure that as large a fraction as possible of the neurons can be stimulated and/or recorded, as well as the inverse, that the largest possible fraction of electrodes report neural signals. Stimulation and recording of neurons in culture depends not only on achieving a satisfactory physical arrangement of neuron and electrode, but also on achieving satisfactory biological maturation so that the neurons are electrically active. The use of micro lithographic patterning techniques can assist in encouraging neurons to grow on top of near to electrodes so as to achieve these aims.

This work shares the general goals of researchers using surface modification and patterning technologies, which include basic understanding of cell biology and neuroscience and the creation of in vitro tissue models for drug screening or cell-based biosensors. It has particular relevance to studies in which the coupling of neurons to electrodes is critical, including neural prosthetic devices, and devices and studies in which patch clamp quality recordings are critical.

The history of MEAs and neural recording dates back to the pioneering work of Thomas et al. [1], Gross et al. [2] and Pine [3]. It has progressed to the point where there are several commercial vendors of MEAs and associated recording equipment for use with dissociated cell cultures as well as brain slice studies. Progress in culture and recording has been made such that there is growing credence to the claim that these cultured neurons retain sufficient pharmacological properties, useful in drug screening and perhaps toxin detectors [4].

The history of the use of lithographic techniques for controlling cell growth dates back to at least the studies of Letourneau [5]. The application to neural cultures was boosted by the pivotal report of Kleinfeld et al. [6] on the use of the microlithographic technology of the semiconductor electronics industry. The field has grown substantially since, with many researchers reporting advances in lithographic techniques, including laser [7, 8], photosist [9, 10] and microcontact printing [11–14] based approaches using a variety of materials and cell populations.

That it is possible to record and stimulate neurons in unpatterned cultures is well established [15–19]. However, such cultures are usually relatively dense in neuron count, strongly overgrown by supporting astrocytes and relatively mature (often months in culture). In contrast, researchers pursuing patterned cultures have set their sights on circuits involving very few neurons, individually identifiable with minimal interference either functionally or optically from other cells including glia. The sparseness of these cultures creates several problems. The cells may not be close enough to electrodes. The population of cells may be insufficient to produce the trophic factors needed to condition the media for survival and activity. The reduced number of supporting cells also may affect survival and activity. Many of the neurons cultured naturally have thousands of input and output synapses, greatly exceeding the available contacts in the sparse cultures.

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Ideally, each neuron would occupy a position exactly on top of an electrode, tightly coupled electrically so as to facilitate stimulation and recording. Indeed, a number of researchers are proceeding on the assumption that gigohm seal, patch clamp-like connections, can be made between cell and electrode. Investigators have started to ask the question as to how close to an electrode a neuron needs to be in order to be recordable. The answer to this question depends on many factors, including extracellular resistivity which is much lower for media in vitro than for brain tissue in vivo, with the effect that in vitro cells must be closer to electrodes than in vivo cells.

This report includes progress toward solutions to both the physical and biological problems in recording from patterned populations of neurons. It illustrates that cellular microlithography has progressed to the point where one can guide neurons over electrodes in both sparse and dense cultures. Absence of signals is unfortunately common, due either to inadequate coupling to electrodes or lack of neural activity. The likelihood of recording action potentials appears to be confined to those neurons whose cell bodies are located either on, or very close to, electrodes. When neural cultures are locally dense, however, it is common to record an abundance of signals. The report here ends by highlighting and interpreting previous work showing that glia are critical to the development of abundant activity in these networks.

The cultures here were created with embryonic day 18 rat hippocampal neurons grown in serum-free media with neither added glia or glia-conditioned media. The media includes a supplement created to optimise neuronal survival, at the expense of glia, at four days after plating, and the cultures appear to be free of glia for approximately the first week. When grown at low density (e.g. 200 cells/mm² or less) it is often possible to follow the development of individual neurons under a light microscope. Cultures this sparse are less robust, however. The earliest that electrical activity may occur is four days, while extracellularly recordable spontaneous activity rarely occurs before ten days to two weeks [20].

2 Methods

The methods have been described elsewhere and are reviewed here in outline form.

2.1 Cell culture

Hippocampal neurons (BrainBits, www.brainbitsllc.com) are taken from embryonic day 18 rats, mechanically dissociated and plated in Neurobasal/B27 medium (Invitrogen, Carlsbad, CA) containing 25 μM glutamate and 0.5 mM glutamine, incubated at 37°C in 95% CO₂ and 5% O₂ gas (backfilled with nitrogen). Each week, one-half of the medium was changed with Neurobasal/B27 medium containing 0.5 mM glutamine.

2.2 Physisorbed substrate preparation

Coverslips (VWR Scientific) or MEAs (Multi Channel Systems (MCS), Reutlingen, Germany) were first sonicated in the acetone bath for 10–15 min and rinsed by isopropanol (IPA) and deionised (DI) water successively. The peak amplitude for 12s; multiple units were sorted manually using template matching or principal component analysis (PCA). Spike waveforms and timestamp data were recorded. The Off-line Sorter (Plexon Inc., TX, USA) was used to record and monitor the continuous raw activity.

2.3 Covalently linked surface preparation

Glass coverslips prepared for covalent linking were first sonicated for 10–15 min in acetone or 10% ethanol in DI and rinsed under running DI for 15 min; they were then cleaned in 70% sulphuric acid in DI overnight, rinsed with running DI for 15 min, and dried with a nitrogen stream. 3-

Mercaptopropyltrimethoxysilane (MPS) was then chemisorbed on to the glass coverslips through 1h long reaction in a 5-10% MPS in distilled methanol reaction mixture (containing 0.5% acetic acid and trace amount of water for catalysis). Following the reaction, the coverslips were dried with a nitrogen stream and baked for 30 min at 120°C. Prior to patterning with polylysine, the silanised coverslips were activated with a heterobifunctional crosslinker (N-γ-maleimidobutyryl-oxysulphosuccinimide ester ( sulpho-GMBS, Pierce), 1 mg/10 ml MES buffer) for 30 min.

2.4 Microcontact printing

AZ4620 (Clariant Corp., Sommerville, NJ) was used to fabricate moulds for PDMS stamps. Patterns used can be seen in the Figures. PDMS was poured in the mould and cured for 6h at 90°C in an oven. Acetone and IPA were used to remove any organic contaminants on PDMS stamps. 10% SDS (sodium dodecyl sulphate) was coated on the stamp surface by soaking it for 15 min [21]. After the SDS coating, the stamp was dried carefully with nitrogen and excess SDS was removed by dipping the stamp once in DI water. Poly-D-lysine (PDL, 0.1 mg/mL, mixed in DI, Sigma) was loaded on the stamp for 30 min. Using a custom-built contact aligner, the stamp was aligned with the MEA or coverslip and brought in contact for 3 min. Printed substrates were rinsed thoroughly with DI water and blow-dried by nitrogen gas.

2.5 Photoresist-assisted patterning

The desired pattern was transferred to the surface of MEA using conventional lithographic techniques. The process involved spin-coating the MEA with AZ5241 (Clariant Corp., Sommerville, NJ), baking on a hotplate at 110°C for 30 s and alignment and exposure to UV through the photomask. After the development of the photoresist, the substrate was hard-baked on a hotplate at 125°C for 90 s and cleaned with oxygen plasma for 2 min. 0.5 mL of PDL solution (0.1 mg/mL, mixed in DI) was applied to the MEA or coverslip and allowed to adsorb for 2h. After PDL adsorption, the substrate was rinsed thoroughly with DI water and the photoresist was removed under an acetone ultrasonication bath for 5 min. The substrate was successively rinsed with IPA and DI water and blow-dried with nitrogen gas.

2.6 Data acquisition and analysis

An MEA was placed in an MEA 1060 amplifier (Gain 1200, 10 Hz–3 kHz, MCS, Reutlingen, Germany) and humidified 5% CO₂, 10% O₂ gas (backfilled with nitrogen) was fed to the recording chamber to maintain the proper pH level. Two different system configurations were used to process 60-channel data from the MEA. One configuration was dedicated to record and monitor the continuous raw data stream using MC Card (sampling rate 25 kHz or 40 kHz) and MC Rack supplied by Multi Channel Systems (Reutlingen, Germany). The other configuration was optimised for real-time spike detection and sorting. In this configuration, the MEA 1060 amplifier was connected with MAP (Multichannel Acquisition Processor, Plexon Inc., TX, USA) and the software package RASPITIN (real-time acquisition systems programs for unit timing in neuroscience, Plexon Inc., TX, USA) was used to process the spike data. Spike detection was done by setting the threshold at four times the standard deviation by collecting the peak amplitude for 12s; multiple units were sorted manually using template matching or principal component analysis (PCA). Spike waveforms and timestamp data were recorded. The Off-line Sorter (Plexon Inc., TX, USA) was optimised for real-time spike detection and sorting.
used for manual spike detection and sorting of the continuous raw data or spike waveform. Subsequent analysis was carried out using Neuroexplorer (NEX, Nex Technologies, MA, USA).

2.7 Microscopy and scanning electron microscope (SEM) preparation

Phase contrast images were taken with an inverted microscope (Olympus IX-51, Olympus America Inc., Melville, NY, USA) using 10x and 20x objectives. In order to get scanning electron microscope (SEM) images, a sample was immersed in 4% paraformaldehyde for 10 min and rinsed twice with phosphate-buffered saline (PBS). PBS was replaced with ethanol by gradually increasing the concentration of ethanol (37% 15 min, 67% 15 min, 95% 15 min). The sample was washed with 100% ethanol for 15 min and repeated three times. Finally, to dry the sample, it was left in HMDS (hexamethyldisilazane) for 45 min and allowed to evaporate. The dried sample was coated with gold/palladium using a sputter-coater (Desk II TSC, Denton Vacuum, Moorsetown, NJ).

3 Results

3.1 Patterning is sufficient to grow cultures with neurons near or on top of electrodes

The question addressed is whether or not patterning is sufficient to guide neurons, whether cell bodies or axonal or dendritic processes, near or over the tops of surface electrodes. Two counterarguments have been raised. Since the surface chemistry of the electrodes is not the same as on the surrounding glass or plastic insulation, neurophilic materials may not bind or absorb to the electrodes. Neural avoidance of electrodes might then result from either dissimilar surface chemistry or changes in the height of the electrode. The resultant pattern could be a diversion of the neural process around the edge of the electrode. In contradiction Chang et al. have shown that platinum, and possibly other metals, can be treated so as to covalently link stamped polylysine. Nam et al. have shown that functional electrode arrays can be made with gold over the insulation and the electrodes, making possible thiol linking of neurophilic materials to both. The addition of cytophobic background materials, such as protein and cell-resistant polyethylene glycol (PEG) can significantly enhance the likelihood of the cells remaining on the neurophilic foreground and over the electrode.

We argue by means of visual examples that patterning is sufficient to bring neurons to electrodes. Figure 1 shows an example of patterned neuron growth in which a 5 μm wide line of polylysine was superposed over the tops of electrodes with the effect that the neural processes appear to have grown exactly on top of a number of electrodes. Figure 2 shows examples from a different culture in which both light microscopic and subsequent SEM images indicate that a patterning has led either a neural fibre or a small bundle of neural fibres to exactly overlay the electrodes. In Fig. 1 the culture was created using a photoresist assisted-patterning described above, while in Fig. 2 the culture was obtained by stamping without the linking chemistry. The images strongly support the concept that the neurons adhere to the recessed electrode surface. Several other images are given below in conjunction with recording experiments. All support the conclusion that culturing procedures and lithography are available to put neurons on top of electrodes.

Fig. 1 Example culture in which neurons were grown in a moat and line pattern
Narrow bundles of neural fibres emerge from moats and grow directly on top of electrodes. Scale: 200 μm centre spacing for electrodes

Fig. 2 Light microscope and SEM images of neural bundles crossing electrodes

3.2 That neurons are near or on electrodes does not ensure recorded activity

The assertion here is obviously true; inactive neurons will not produce recordable signals. Still, this simple truth is the source of much consternation among researchers developing new systems, where it is difficult to identify whether the lack of signal originates from biology or an instrumentation problem. Chang et al. reported experiments, both patterned (40 μm and 12.5 μm wide lines) and unpatterned, in which 93% of electrodes had a nearby soma or neurite, yet less than a third of the electrodes reported action potentials [24, 25]. An example is given here to illustrate that there are times when signals are expected and still not recorded. Figure 3a is an optical image of a bundle of neurons traversing sequentially four electrodes.
with sufficient geometric overlap of the electrodes that one would predict recordings on all four sites. Figure 3b shows electrical recordings from these four electrodes, clearly indicating strong signals on channels 1 and 4, weak signals on channel 2 and no signals on channel 3. The differing duration of the bursts indicates that different sets of neurons were recorded among the electrodes, although it is possible that some of the signals are axonally conducted action potentials. The absence of spikes on channel 3 is surprising. Figure 3a suggests that recordable signals arise when somata are on top of the electrodes (channels 1, 2, 4) but not otherwise (channel 3). Although the SEM images (Fig. 3c) show some damage due to fixation, they nonetheless convey the impression of small clusters over electrodes 1 and 4 and to a lesser extent 2, but not 3. This suggests the possibility that the strongest signals arise in part due to an insulating cap of glia overlying both neurons and the electrode. This would cause more localisation of action currents and hence larger signals at the recording site.

### 3.3 Somata must be on or close to electrodes if signals are to be recorded

Figure 4 shows images of two neurons in unpatterned culture that lie either on or close to the electrodes. They were part of an experiment summarised in Table 1 in which all electrodes were examined for the presence or absence of both electrical signals and nearby neurons. We conclude that when cell bodies lie directly on top of electrodes, their action potential signals can be recorded, but that not all cells may be active; here two-thirds of the electrodes are active. The action potentials from cells within perhaps 10 μm can also be recorded (here approximately one-third of the cells within a 20 μm radius are recordable), but not when the cells are much further away. Since the resistivity of the cell culture media is one-fifth that of brain tissue [26], this extrapolates to being able to record action potentials in the brain from individual cell bodies that lie within approximately 50 μm of the electrode, an estimate that is consistent with reports in the literature [27].

### 3.4 Growing many neurons near electrodes is sufficient for abundant recording activity

Figure 5 shows an experiment utilising 100 μm diameter cytophilic regions surrounding the electrodes, with the result that abundant activity was detected after 11 days. A total of 92 neural units were detectable on the 60 electrode channels. Figure 6 shows a pattern of 40 μm wide lines where many

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**Fig. 3** Neural bundle grown over the top of four array electrodes

a Transmitted light microscopic image of living culture (200 μm centre spacing of electrodes)

b Electrical traces (full scale +/− 100 μV; 5 sec)

c SEM photos of the neurons in (a) after fixation

**Fig. 4** SEM photos of neurons whose cell bodies grew either on top of or very near to electrodes

Electrode diameter is 30 μm

**Table 1:** Fraction of electrodes with detectable spikes according to the location of neurons relative to the electrode

<table>
<thead>
<tr>
<th>% of Electrodes</th>
<th>Direct contact</th>
<th>Near (20 μm radius)</th>
<th>Far</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>20</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Inactive</td>
<td>10</td>
<td>12</td>
<td>53</td>
</tr>
</tbody>
</table>

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neurons overlapped electrodes after 12 days in culture, with abundant activity as shown in Fig. 7. Both experiments indicate that recordable activity increases with local density in the vicinity the electrode, probably for both geometric (proximity) and biological reasons.

3.5 Importance of glia in maturation of neuroelectrical activity

In previously reported work it was shown that restriction of neurons to relatively narrow lines (40 µm and 12.5 µm wide) increased the electrical activity of the neurons in the network compared to cultures of the same initial density in which the substrate had a uniform coating of polylysine [22, 25]. Further, the confined networks showed earlier maturation of glia, predominantly astrocytes and synapses [22, 28]. These cultures had virtually no glia after four days in culture [29], so the rapid subsequent growth of glia was surprising. Still, it led to the conclusion that glia were essential to the health and activity of the neural networks, especially since the culture is sparse and highly sensitive to growth conditions. Table 2 reanalyses some of the data from these reports. It shows that neural growth was denser and presumably healthier in the confined patterns. Further, there is evidence of more rapid maturation of glia and synapses on the cultures with patterns. Interesting also is that the glia come to outnumber the neurons in approxi-

Fig. 5  

a Culture seeded using 100 µm diameter circles over electrode arrays  
b Action potentials were recorded from 92 different neural units from (a)
mately two weeks and begin to assume ratios suggestive of the ten to one ratio in the brain [30]. The number of synapses per neuron also approaches physiological levels despite the greatly reduced number of target neurons on which synapses can be made.

4 Discussion and conclusions

The data and images shown here strongly support the notion that the position of neurons in culture can be controlled so as to greatly enhance the likelihood that recordings can be made using surface microelectrode arrays. The basic element is microolithography of a cytophilic material, such as polylysine as was used here. Process features that improve pattern fidelity are covalent linking of the cytophilic material to the surface and the use of a protein-resistant and hence cytophobic background material, such as polyethylene glycol. Provided that pattern alignment is precise, somata and extremely fine bundles of processes, perhaps down to 1 µm in diameter, can be localised to the electrodes. We argue, by way of multiple examples, that neurons can be routinely coupled to electrodes.

Localisation of neurons is a necessary, but not sufficient, condition for the recording of action potential activity. As shown in Table 1, it appears that a soma can be no further than 10 to perhaps 20 µm for recordability. This extrapolates by a factor of five to 50–100 µm in vivo in the brain, a figure consistent with the literature [27]. No data were shown demonstrating that one can record from either dendrites or axons in this system. Presumably the currents from these processes are much smaller than from somata, resulting in smaller extracellular potentials and the need for even better coupling to the electrodes. As hinted at in Fig. 4, the recording of axonal spikes may require an overlay of insulating glia in order to confine current paths sufficiently that the electrodes will detect action potentials above the noise level. Dense but localised cultures, such as are shown in Figs. 5 and 6, are much more likely to have activity.

Our previous reports indicate that glia are an essential part of neural cell culture. Conditions that favour the growth of glia enhance the development of neurons and their spontaneous electrical activity. The local density of neurons and convergence of synapses are correlated with enhanced glial development and enhanced electrical activity in patterned cultures. Hence, patterning can enhance not only the likelihood of electrical coupling to electrodes but also the existence of electrical activity.

5 Acknowledgments

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6 References


Table 2: Companion of neural, glial and synaptic densities in unpatterned and patterned cultures

<table>
<thead>
<tr>
<th>Day in culture</th>
<th>Neurons (per mm²)</th>
<th>Glia/neuron ratio</th>
<th>Synapse/neuron ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unpatterned</td>
<td>Patterned</td>
<td>Unpatterned</td>
</tr>
<tr>
<td>8</td>
<td>150</td>
<td>270</td>
<td>0.3</td>
</tr>
<tr>
<td>15</td>
<td>140</td>
<td>300</td>
<td>1.0</td>
</tr>
<tr>
<td>22</td>
<td>80</td>
<td>150</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Data reinterpreted from Chang [24]