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Electrical stimulation and recording from cultured neurons using a planar electrode array

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Abstract

Planar electrode arrays were fabricated using modern semiconductor technology. Mouse and chick dorsal root ganglion cells were successfully cultured on the surface of indium tin oxide electrode patterns. Contact between the neurites and the substrate electrodes was established by exploiting the surface properties of the culture substrates to guide neurite outgrowth. The guided neurites were stimulated electrically using the substrate electrodes and cell responses were recorded intracellularly and extracellularly. Depolarization of the cell membrane as well as generation of action potentials in response to the stimulation could be observed. The results suggest that this type of planar electrode array could be a useful tool for non-invasive electrophysiological measurements.

INTRODUCTION

The architecture of neuronal information processing can be perceived as being organized in three layers: cellular processing, communications between cells and functions expressed by whole networks. Measurement of electrophysiological characteristics is necessary for the understanding of each of these layers. Experimentally, simultaneous monitoring of both morphology and electrical activity is desirable. However, the nervous system in vertebrates is so complicated that in-vivo monitoring, particularly of multiple neurons, is difficult. One promising method of circumventing this problem is to use dissociated cell culture. Neurons survive for several weeks in culture and reorganize into networks with a two-dimensional distribution of cells; individual neurons as well as their connections are visible in culture dishes. In addition, cultured neurons maintain most of their in-vivo

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biochemical, electrophysiological and morphological characteristics. Thus cell culture provides a very useful method for studying the physiology of neuronal systems.

Glass pipette microelectrodes have been most widely utilized for electrophysiological measurements in culture. It is possible to obtain tip diameters of less than 1 μm and to record the intracellular potential directly. Glass microelectrodes have two limitations, however, one is that they must be inserted into the cell. Because of the damage caused by the insertion, glass microelectrodes are not suitable for long-term monitoring of cell activity. The other limitation is the difficulty of handling multiple electrodes. Multisite recording and stimulation is essential for analysis of cellular processing and network functions, and requires the attachment of multiple electrodes simultaneously. The precise positioning of each glass pipette becomes prohibitively difficult for more than a few electrodes. Because of these limitations, glass microelectrodes are not adequate for long-term multisite analysis of cultured neural systems. The polished metal microelectrode is another candidate for non-invasive extracellular recording and stimulation, but the multiple electrode problem is the same as for glass microelectrodes.

In this work the feasibility of using substrate electrodes was studied. Multiple electrodes of less than 10 μm can easily be patterned on substrates with good reproducibility by photolithography. The application of needle-type electrode probes fabricated by semiconductor technology to recording of cell activity has been reported [1–3]. Substrate electrode have also been applied to cultured cells [4–8]. However, the positions of the cells could not be controlled in these studies, so that good contact between cells and electrodes was rarely established. In our study this problem was solved by guiding neurite outgrowth towards the electrodes by using the surface properties of the culture substrates. Neurite outgrowth guidance has been extensively studied [9–16]. Both differences in cell adhesion to materials and the geometry of the surface structure of the substrates were used in our work. This controlled outgrowth enabled us to stimulate neurites using the substrate electrodes and to record membrane potential changes corresponding to the stimulation. Long-term recording of activity from neural networks in culture is also feasible using this method.

EXPERIMENTAL

Design and fabrication of electrode arrays

Figure 1 shows the process of fabrication of electrode array substrates for cell culture. Quartz was used as the insulating substrate. Quartz coated with 100 nm sputtered indium tin oxide (ITO), a transparent conductive material [17], was purchased from Central Glass Co. Ltd., Tokyo. First, ITO was etched in HCl solution and the electrode pattern was formed. Then the surfaces of the ITO electrodes were partially plantinized electrochemically. The purpose of this procedure is to decrease the interface impedance between the electrode and the electrolyte solution. After that, aluminum oxide was deposited onto the substrates

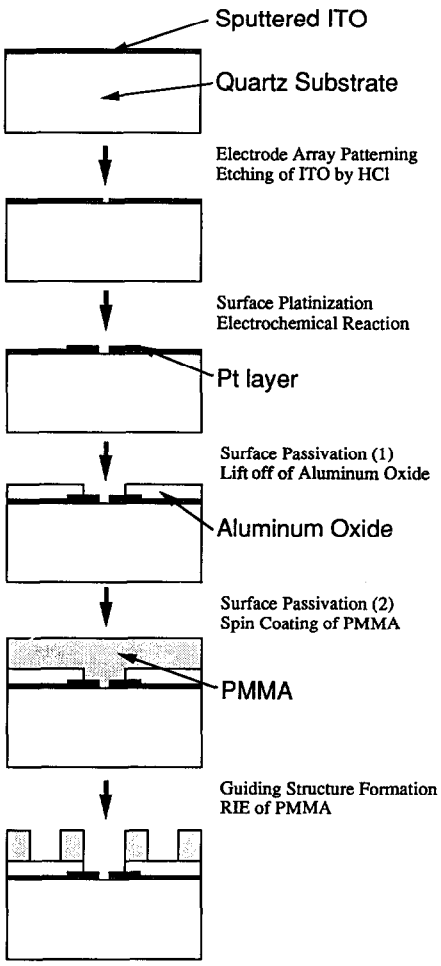

Fig. 1. Fabrication process for the planar electrode arrays. The process consists of the following three steps: electrode array formation, deposition of a passivation layer and construction of the guiding structure.

as the passivation film, except at the very tip of the electrodes. Finally, a poly(methyl methacrylate) (PMMA) film was spin-coated and patterned by reactive ion etching (RIE). Using RIE, the edge of the PMMA film was etched sharply so that we could obtain clear boundaries to the grooves. The thicknesses of the insulating films were 700 nm (aluminum oxide) and 1 μm (PMMA).

Figure 2 shows photographs of the electrode array substrate. The size of the opened electrode site was 10 μm $\times$ 10 μm. Electrodes were used in pairs to restrict current flow during stimulation and to allow differential measurement for recording. We designed a pattern with 18 pairs of electrodes (36 electrodes). A

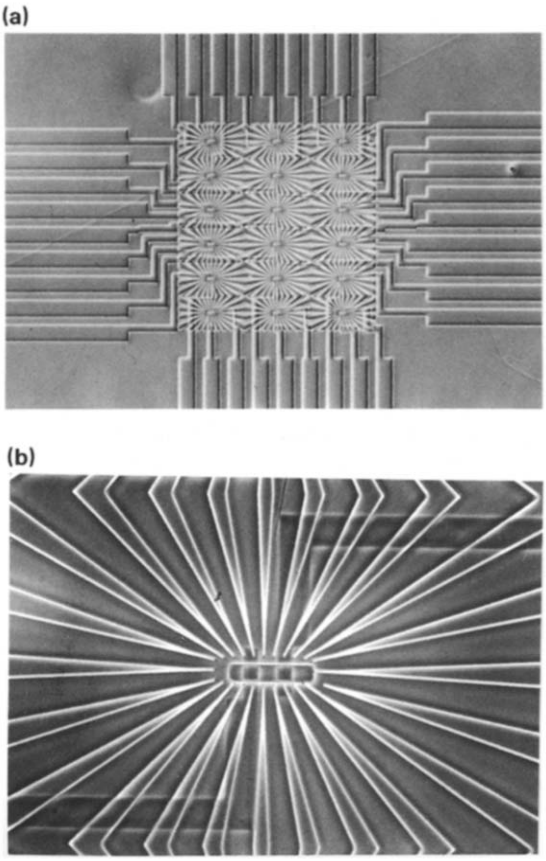

Fig. 2. (a) A phase contrast micrograph of the electrode array pattern. There are 18 pairs of ITO electrodes. The guiding structure can be seen clearly. (b) A magnified view of the center region of the electrode array substrate.

magnified view is also shown in Fig. 2. Aluminum oxide and PMMA served both as insulators for passivation as well as guiding structures for neurite outgrowth, as will be described later.

Cell culture

Adult male mice (6 weeks) or chick embryos (14–17 days) were used. The dissection procedure was as follows: (1) dissection of the dorsal root ganglia (DRG); (2) digestion by collagenase (Boehringer) in Leibovitz's L-15 medium (Gibco) at 37°C for 1 h; (3) trituration using a pipette; (4) seeding on electrode array substrates.

TABLE 1

Composition of the culture medium

Chemicals	Concentration
Ham's F-12	50%
Dulbecco's MEM	50%
Progesterone	20 nM
Insulin	5 μg/ml
Transferrin	30 μg/ml
Putrescin	100 μM
Na_2SeO_3	30 nM
7s NGF	20 ng/ml
Laminin	10 μg/ml

The culture medium consisted principally of 50% Ham's F-12 and 50% Dulbecco's MEM (Gibco). Progesterone, putrescine, insulin, transferrin (Sigma), 7s nerve growth factor (WAKO) and laminin (Boehringer) were also added, in the concentrations given in Table 1.

Cell cultures were maintained in a 37°C, 5% CO_2, water-saturated atmosphere, and used for measurements after 3 or 4 days of incubation.

Electrophysiological measurements

The neurons cultured on the electrode array substrates were stimulated electrically by the substrate electrodes and the corresponding cellular response was recorded intracellularly by a glass microelectrode and extracellularly by the substrate electrodes. Figure 3 shows a schematic diagram of the measuring system. Electrical pulses were transferred into substrate electrodes through an optically coupled isolating circuit. Intracellular recording was performed using the whole-cell patch clamp method [18]. TW150-4 capillary (WPI) was used to make the glass microelectrodes. An Axopatch-1C (AXON instruments) and a DAM80 (WPI) were used as the amplifiers for intracellular and extracellular recording respectively. Stimulating pulses were controlled by a personal computer through an IEEE 488 interface. The recorded signals were also analyzed using the personal computer.

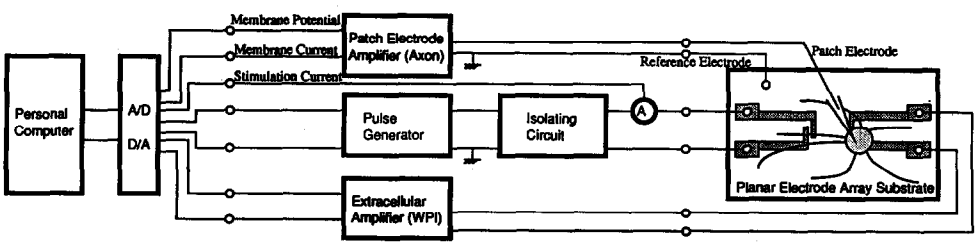

Fig. 3. A schematic diagram of the electrophysiological measuring system. The apparatus includes a computer for system control and data analysis.

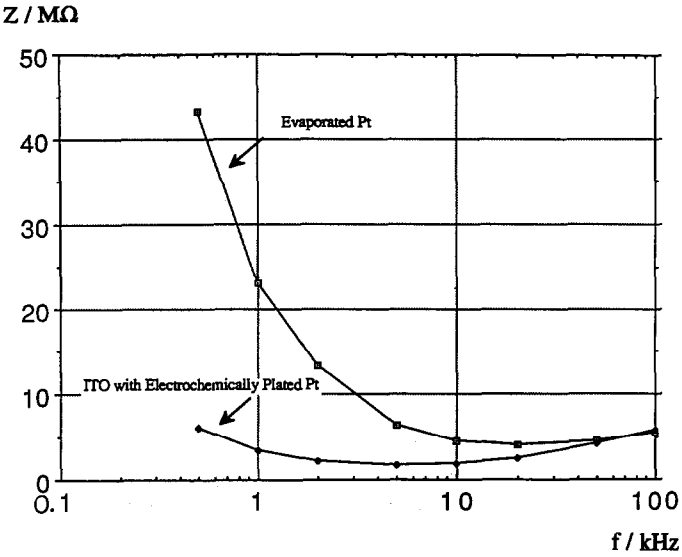

Fig. 4. Dependence of electrode impedance on frequency. Comparison of bare ITO electrodes with those with an electrochemically coated Pt layer.

RESULTS AND DISCUSSION

Characteristics of electrodes

Electrode impedance is determined by the size of the electrode and the properties of the interface between the electrode and the electrolyte solution. The size of the stimulating site of the substrate electrodes is 10 μm $\times$ 10 μm. If the interface impedance is too large, the passivation layer is electrically broken when a stimulating pulse is applied. Also, excess input impedance causes a reduced signal level in recording. Taking these factors into account, it is desirable that the input impedance of electrodes be kept below a certain well-defined criterion. In our study, electrochemical surface platinization was carried out. Figure 4 shows the frequency dependence of the electrode impedance. The impedances of evaporated Pt electrodes and of ITO with electrochemically plated Pt were compared. All electrode pairs were measured and their average was plotted. The results show that electrochemical plating of Pt produced a definite decrease in interface impedance, particularly in the low frequency region. The impedance of electrochemically Pt-plated electrodes was of the order of a few megaohms at 1 kHz.

Guidance of neurite outgrowth

Neurites regenerate in the culture dish. Under normal conditions, however, the direction of outgrowth is random. Therefore the neurites rarely contact the

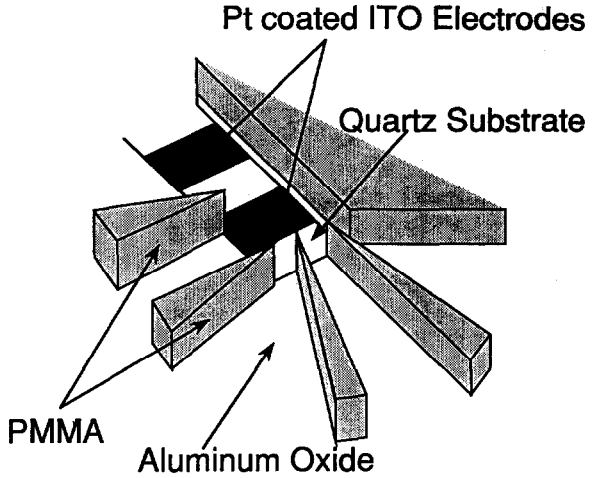

Fig. 5. A schematic diagram of the surface microstructure of the electrode array substrate. The electrodes were coated with aluminum oxide passivation film. A PMMA wall was constructed on the film to guide neurite outgrowth.

substrate electrodes. In order to improve this situation, it is necessary to develop some method of establishing neurite–electrode contact.

Neurite outgrowth is guided in vivo by chemical substances referred to as trophic factors. In vitro, however, it is difficult to maintain a concentration gradient of these materials over a long period. We studied the dependence of neurite outgrowth on the surface properties of culture substrates in culture. The results can be summarized as follows.

(1) Neurites recognize the microstructure of the culture substrates. On a substrate with microwalls and microgrooves, once neurites drop into the grooves they seldom grow out over the walls [15].

(2) Neurites prefer some materials to others. The adhesiveness of neurites to substrates depends on the substrate material. Neurites prefer some organic substances such as laminin. However, a major technical limitation is that the substance must retain its original properties after the photolithography process. Therefore we chose metal oxides as stable inorganic materials. Among the six metal oxides tested [16], we found that aluminum oxide was the most suitable material since it was non-toxic to neurons while also providing enough adhesiveness for neurites. We designed substrates with a surface structure as shown in Fig. 5, which consisted of microgrooves with aluminum oxide surfaces and walls made of PMMA.

Figure 6 shows the results of culturing neurons on these designed substrates. It can be seen that neurites contact the aluminum oxide surface and that their outgrowth is guided by the PMMA walls. The neurites are successfully guided towards the substrate electrodes.

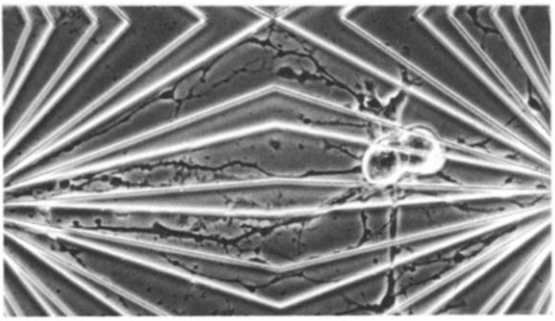

Fig. 6. Cultured mouse DRG neurons on the planar electrode array substrate. Neurites grow along the guiding structure.

Electrical stimulation

Figure 7 shows an example of electrical stimulation by the substrate electrodes. The three curves are trajectories of membrane potential recorded with the intracellular electrode. The intensity of the stimulating current pulse of duration 0.5 ms was gradually increased and the corresponding cell responses were recorded. Curve A was obtained with the most intense stimulation and curve C with the least. In curve C, we can see only artifacts caused by the stimulation pulse. In curves A and B, however, a shoulder peak is observed. The two traces inserted in

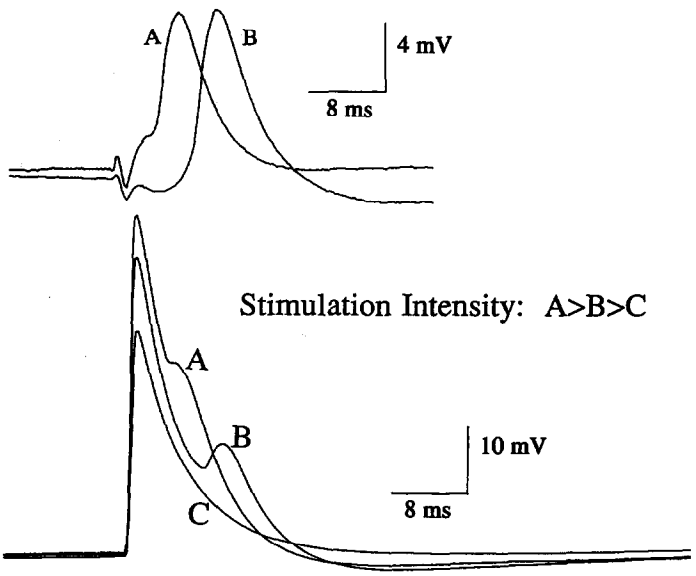

Fig. 7. Membrane potential with subthreshold stimulation. Latency of the cell's response depended on the stimulation intensity.

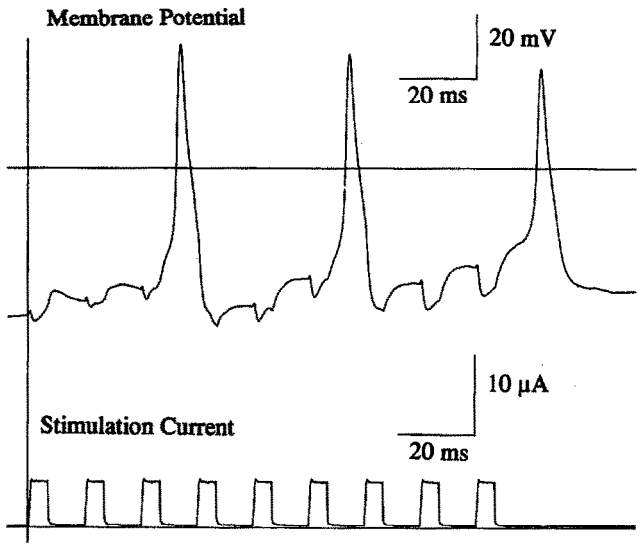

Fig. 8. Cell response to a train of current pulses. An action potential was generated after every third stimulation pulse.

the figure are obtained by artifact subtraction – a trace obtained at low stimulation intensity showing only artifact was scaled appropriately and then subtracted numerically from the measured data. After subtraction, the shapes and amplitudes of the peaks in the two traces are almost the same. Only the latency changed with stimulation intensity, decreasing as the stimulation intensity was increased. These results most probably reflect all-or-none opening of voltage-dependent ion channels at a certain threshold voltage, which is reached after shorter times with stronger stimulation.

The cellular response to a train of stimulating pulses is shown in Fig. 8. A train of pulses of 5 ms duration was applied. In this case, action potentials were generated at every third stimulating pulse, implying that a single pulse of this intensity was not enough to depolarize the membrane to the threshold voltage for action potential generation but that a train of three pulses produced enough depolarization for impulse generation through temporal summation.

These results indicate that our planar electrode arrays could be useful tools for the electrical stimulation of cultured neurons. Controlled outgrowth enables us to stimulate particular neurites, providing an experimental method for studying the signal conduction process in neurites. If neurons were seeded at particular positions at the beginning of cell culture, then it would be possible to stimulate particular cells in the network. Such cell-selective stimulation in an artificially guided neural network should be the next step in the development of this technique.

Cell activity recording

The substrate electrode can also be used for recording in addition to stimulation. Figure 9 shows an extracellularly recorded signal when a neurite is stimulated using another substrate electrode pair. The configuration of the stimulation

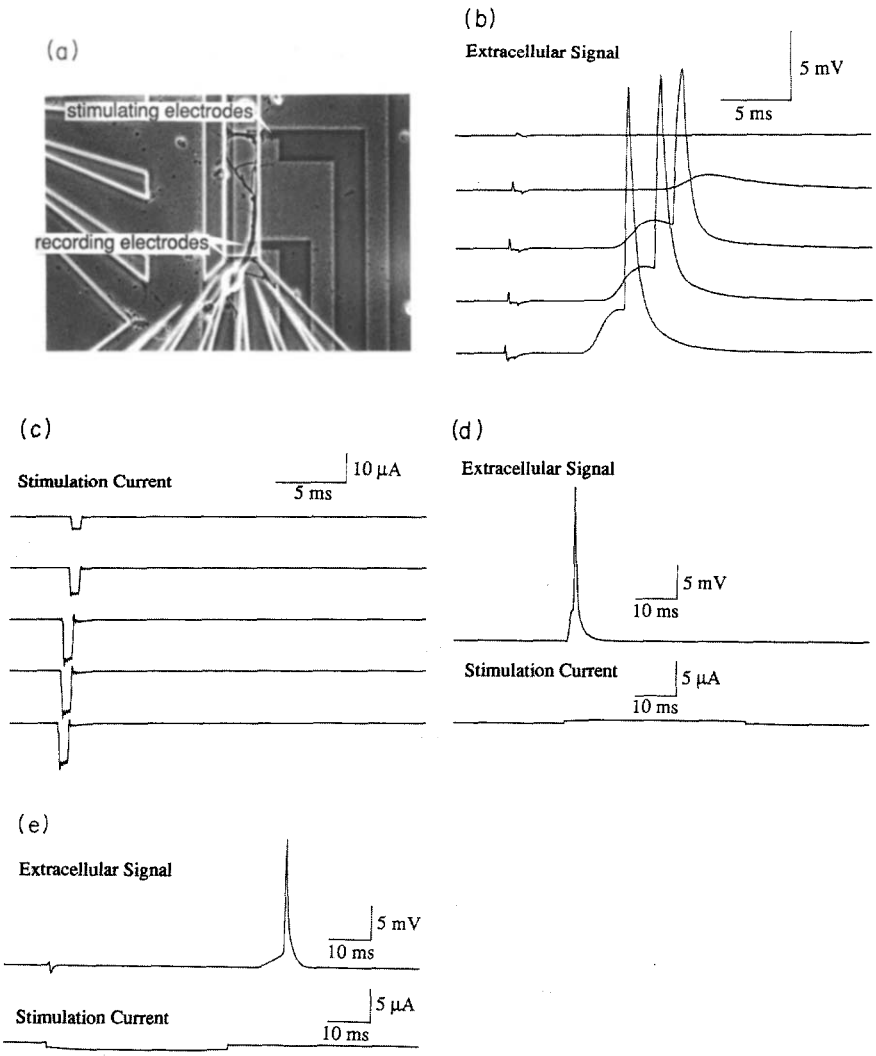

Fig. 9. Extracellularly recorded cell activity: (a) configuration of stimulating and recording electrodes and the cultured neuron; (b) cell response when the stimulation current was gradually increased; (c) corresponding stimulation current. When the polarity of the stimulation pulse was inverted, the cell's response timing changed like an "on and off reaction", as shown in (d) and (e).

electrodes, the recording electrodes and the neuron is shown in Fig. 9(a). The neurite grows along the guiding structure and makes contact with the electrodes.

Figures 9(b) and 9(c) show the results when the stimulation intensity was gradually increased. Both subthreshold depolarization and impulse generation were observed, depending on the stimulation intensity. The value of the extracellularly recorded signal (around 10 mV) was very high, which suggested that an almost gigaohm seal was established in this case. Figures 9(d) and 9(e) are the results obtained when the polarity of the stimulation pulse was inverted. A phenomenon similar to the "on and off reaction" was observed. This type of extracellular recording using substrate electrodes is a non-invasive method and permits long-term recording of cell activity.

CONCLUSIONS

Effective and localized electrical stimulation as well as extracellular recording from cultured neurons was achieved using planar electrode arrays. Controlled outgrowth of neurites by the surface microstructure of the culture substrates was used to stimulate particular neurites and record the corresponding cellular response.

The latency of membrane depolarization was observed to vary with stimulation intensity. A stimulation pulse train caused generation of action potentials by temporal summation. Membrane depolarization as well as impulse generation could also be recorded extracellularly by the substrate electrodes. These results indicate that our planar electrode arrays should be useful tools for electrophysiological measurement. Further development of this method will involve long-term monitoring of network neural activity and cell-selective stimulation.

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