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Technical steps towards one-to-one electrode–neuron interfacing with neural circuits reconstructed in vitro

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Abstract

The electrical interfacing with cultured networks of neurons by means of multielectrode arrays (MEAs) is an area of intensive research given the potential of this technique to aid in the extraction of the algorithms that support neurocomputation in brain tissue. To this end, we have previously described the polymer-on-multielectrode technology (PoM [E. Claverol-Tinturé, M. Ghirardi, F. Fiumara, X. Rosell, J. Cabestany. Multielectrode arrays with elastomeric microstructures for neuronal patterning towards interfacing with uni-dimensional neuronal networks. J. Neural Eng. 2(2) (2005) L1–7] and demonstrated single-site recordings from patterned invertebrate cells. The realisation of the full potential of PoM is dependent on its successful application to vertebrate cells and to the recording of activity in ensembles of neurons. Here, we describe progress in these directions, specifically towards the development of vertebrate neuronal cultures devoid of glial layers compatible with PoM devices, the connection of pairs of invertebrate neurons threading microchannels and the recordings of synaptic and spike-like activity.

Keywords: Neural interfaces; Multichannel electrophysiology; Neuronal culture; Cell patterning; Microfluidics; Snail neurons; Helix aspersa; PDMS

1. Introduction

Neurocomputation as implemented by aggregates of living neurons depends on the electrical interplay of large numbers of synaptically connected cells. To achieve a better understanding of network level information processing phenomena, it is advantageous to build realistic computer models. These rely heavily on detailed physiological data describing neuron–neuron interactions [9] and, therefore, are in need of experimental techniques suitable for monitoring neuronal activity in networks of neurons.

Conventional multielectrode arrays (MEAs) are capable of multi-neuron electrical recordings [7,11,13] typically by means of multiple microelectrodes embedded in a flat substrate on which neurons can be grown to form networks. In the most common experimental configuration, neurons are cultured in large quantities (of the order of 5×10^4) and are monitored by up to 100 electrodes. The obvious difference in number between cells and recording sites makes one-to-one neuron–electrode interfacing problematic and, as a result, MEAs have fallen short of the expectations created by the first developments in the area.

To realise one-to-one interfacing, every neuron must be grown in the proximity of a single electrode with a pre-specified geometry because the position of the recording site with respect to the cell body and processes (dendrites and axons) influences greatly the magnitude and shape of the recorded signal [5].

Significant progress towards neuron patterning on MEAs has recently been achieved employing either chemical or physical guidance of cell growth. Chemical strategies rely on the selective coating of cell culture substrates with promoters or inhibitors of cell adhesion and growth. A popular realisation of this approach is the microcontact printing of adhesion molecules such as poly-L-lysine [3] by physical contact of a pre-soaked rubber stamp on a substrate. While this technique is relatively straightforward, issues of long-term cell compliance and

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clustering remain. On occasions, typically after days or weeks of culture, neurons can cross over areas coated with adhesion inhibitors, loosing compliance with the pre-defined growth pattern. Moreover, while chemical strategies can force neuron growth over specific regions, within these areas the continuous movement of cell bodies remains difficult to restrain, often resulting in cell aggregation and precluding one-to-one neuron–electrode interfacing.

A second family of cell patterning techniques addresses the issues of compliance and clustering by means of physical confinement of neuronal growth. To this end, 3D structures of dimensions in the order of tens of micrometres are produced using well established microfabrication techniques so that cell growth is confined by solid features such as walls and pillars. Along these lines several proof-of-principle results have been achieved over the last decade using microwells in silicon [10], Parylene cylindrical cages [15] and SU-8 pillars to form fences [17]. Yet, this success has not resulted in widespread adoption of microfabrication-based cell patterning techniques on MEAs partly due to the high complexity and cost of the device production procedures involved.

To address this issue, we are pursuing an experimental programme towards novel devices capable of physical cell guidance and extracellular recordings while remaining suitable for production at moderate costs. We have previously described the polymer-on-MEA (PoM) technology [4] which combines polymeric films containing microwells and microchannels fabricated with soft-lithography techniques [8,16] and standard planar MEAs and achieved physical confinement and guidance of individual mollusc neurons in the proximity of recording sites and demonstrated multisite extracellular recordings.

However, to realise the full potential of PoM, its optimisation for the monitoring of ensembles of interconnected neurons, rather than isolated cells, would be advantageous. Moreover, culture of vertebrate neurons would be preferable to invertebrate ones as the former could support the study of high-level cognitive functions.

Here, we describe progress towards these two goals: (1) demonstrating the culture of vertebrate cells devoid of glial carpet (non-electrogenic companion cells that facilitate neuron survival but can insulate the electrodes and deteriorate the electrical signals), (2) patterning both vertebrate and invertebrate neurons by polymeric films and (3) recording electric potentials of synaptic origin produced by pairs of interconnected neurons.

2. Methods

2.1. Vertebrate neuron culture

The protocol used for culturing cortical neurons was similar to those described previously [1,2,12] with some modifications. The frontal cortex of Sprague–Dawley pups at stages P1–P7 was dissected and pieces of approximately 2×2 mm cut with a scalpel. The dissection was performed in L15 at 4 °C. Digestion with papain (12 units/ml in L15) was performed at 37 °C for 30 min in a 15 ml tube on a rotating platform. The tissue was then rinsed thoroughly with dissection medium and mechanically dissociated by 40 suction/expel cycles with a plastic pipette tip cut to an opening of 2 mm in diameter. The cells were plated on poly-L-lysine treated Petri dishes or glass coverslips (0.1 mg/ml for 24 h) in neurobasal supplemented with B27 and glutamine and kept at 37 °C in an atmosphere of 5% CO_2. Serum was not used in order to control growth of glial cells. This protocol sufficed to achieve healthy cultures for up to 2 weeks.

Recently, a coculture step [1] has been introduced in order to facilitate long-term survival. Glass coverslips with neurons cultured for 2 days as described above were turned upside-down (cell bodies facing down) and dropped on a glial feeder layer which had been cultured for 10 days to reach confluency. The protocol to culture this layer on a glass coverslip is identical to that described for neurons with the exception that 10% horse serum (Sigma) was added to the culture medium. As a result, the bottom layer was rich in glial cells and poor in neurons and was close enough to the top layer, where glia was absent, to deliver the factors that support long-term survival of the neurons.

2.2. Invertebrate neuron culture

Culture of Helix aspersa neurons followed procedures described previously [4,6,14]. Briefly, snails were collected locally or provided by a breeder (Cal Jep, Spain) and kept in hibernation until wetted prior to the dissection. An active snail was selected and anaesthetised by injection of 1–2 ml of 0.08 mM $MgCl_2$. The circum-oesophageal ring was removed, pinned on cured silicone, trimmed free of surrounding tissue and cut into eight pieces with fine scissors. Using a plastic pipette, these were transferred to a 35 mm Petri dish with culture medium (40% L15 Leibovitz medium supplemented to the following concentrations: 62 mM NaCl, 5 mM KCl, 7 mM $CaCl_2$, 5 mM $MgCl_2$, 20 mM HEPES, 10 mM D-glucose, peniciline 100 units/ml, streptomycine 100 mg/l, fungizone 2.5 mg/l, adjusted to pH 7.6 with NaOH). The ganglia were then torn with hypodermic needles, resulting in the release of multiple neurons from the neuropiles. After a 20 min settling period, healthy cells were identified by their round shape (intact membrane) and transparent/bright cell body as seen under the dissection microscope. Cells with a diameter of 20–50 μm that had survived the procedure were sucked with blunt-opening capillaries (0.58 mm ID, WPI, USA) and expelled into a Petri dish with filtered medium. This cleaning step was repeated twice with two additional dishes filled with clean medium in order to limit the amount of debris and microorganisms carried over from the snail to the PoM device. Finally, the neurons were dropped inside PoM wells.

2.3. Fabrication of PoM devices

The fabrication of PoM devices has been described previously [4]. Briefly, indium tin oxide (ITO) is sputtered on glass substrates (resistivity 80 Ω/sq) or purchased with similar characteristics (PGO, Germany). A set of 18 parallel electrodes, 40 μm in width, are defined by standard photolithography techniques. The substrates are sonicated in acetone, isopropanol, neutral detergent and milli-Q grade water before assembly with elastomeric overlays. The overlays are polydimethyl siloxane (PDMS) films (60–80 μm thick) with microwells and microchannels fabricated by microhole punching and soft-lithography using SU-8 masters [4] and laid on substrates manually with only coarse alignment. The substrate was treated with poly-L-lysine for 24 h prior to overlaying of the PDMS films in order to promote cell adhesion and growth.

2.4. Data acquisition and analysis

A 16-channel amplifier (gain $\times 1300$, bandwidth 3.4 KHz) made contact to the PoM device through an anisotropic conductive film (Shinetsu, Japan). The amplified signals were digitised with a 12 bits ADC (6804E, National Instruments, 10 Ksamples/s per channel) and stored for offline analysis. Of the 18 electrodes present in the device, numbers 1 and 18 were connected to the ground of the circuit and the remaining sites used for recoding. Line interference at 50 Hz and harmonics up to 350 Hz were filtered out using Matlab (Mathworks, USA).

2.5. Pharmacological stimulation

Recordings were attempted from neurites extending beyond 100 μm into a microchannel since previous experience suggested that these are capable of generating spikes above typical noise levels. If spontaneous firing was not observed, stimulation by raising the extracellular concentration of KCl from 5 to 25 mM was usually effective.

3. Results and discussion

3.1. Glia-free cultures from rat frontal cortex

PoM devices include microchannels with a cross-section of 16×6 μm that guide neurite growth. The height of these conducts (6 μm) is adequate to allow threading by individual dendrites and axons of both vertebrate and invertebrate neurons. However, vertebrate neuronal cultures often include a glial cell carpet on top of which neurons develop and whose thickness can vary over time, potentially blocking the microchannels. Moreover, glial cells extend on recording sites and can affect the effective electrode impedance, acting as insulating sheaths. The arrows in Fig. 1A indicate the limits of a patch of glial carpet grown in 10% horse serum (see methods).

In the context of PoM research, it would be therefore advantageous to achieve cultures of vertebrate neurons devoid of glial cells. By eliminating the use of serum, often used as supplement in culture medium, glial cells are virtually eliminated. Figs. 1B–F show neurons grown on a plastic substrate after 7 days. Fig. 1B illustrates how the cells cluster and establish long-ranging connections without a glial layer while, at lower densities (C–F), individual neurons remain identifiable and the probability of clustering decreases.

In agreement with previous work by others [1], we observed that the neurons cultured without glia, while showing extensive dendritic and axonal growth and the formation of networks, were relatively short-lived (approx. 2 weeks). As long-term survival could be important for studies of learning with PoM devices, we adapted a coculture technique in order to provide the neurons with glia-produced factors that support long-term survival. Figs. 2A and 2B show neurons 4 weeks after plating, in isolation and forming networks, respectively. These preliminary results indicate that coculturing is a valid strategy for enhancement of vertebrate neuron survival in the absence of glia.

3.2. Biocompatibility of PDMS films and patterning of vertebrate neurons

In parallel with the studies on glia-free cultures of vertebrate neurons, the biocompatibility of polymeric films and their ability for patterning, was essayed. Fig. 3 shows images of a PDMS film, similar to those used in PoM devices, patterning rat frontal cortex neurons cultured with serum. A microwell (left of Fig. 3A and Fig. 3B) was populated with neurons which grew processes (arrows in Fig. 3B) along a microchannel. The top-right angle of image 3A shows a population of mixed neuronal and glial cells and the distal end of the conduct guiding neuronal processes from the well to the culture beyond the film.

The anatomically healthy cultures obtained within and around polymer films suggest their compatibility of vertebrate neurons and their effectiveness at guiding their processes.

3.3. Connection of pairs of Helix aspersa neurons in PoM devices

While growth of individual invertebrate neurons within PoM devices and extracellular recordings have been demonstrated [4], the connection of pairs of neurons had not been achieved. Fig. 4 shows progress in this direction. Two neurons were positioned within two microwells separated 320 μm and interconnected by a microchannel. After 7 days in vitro, outgrowth along the channel made possible anatomical contact between the two cells (top image in Fig. 4). Extracellular recordings from intra-channel sites are shown (bottom). Work is in progress towards the development of data analysis tools based on

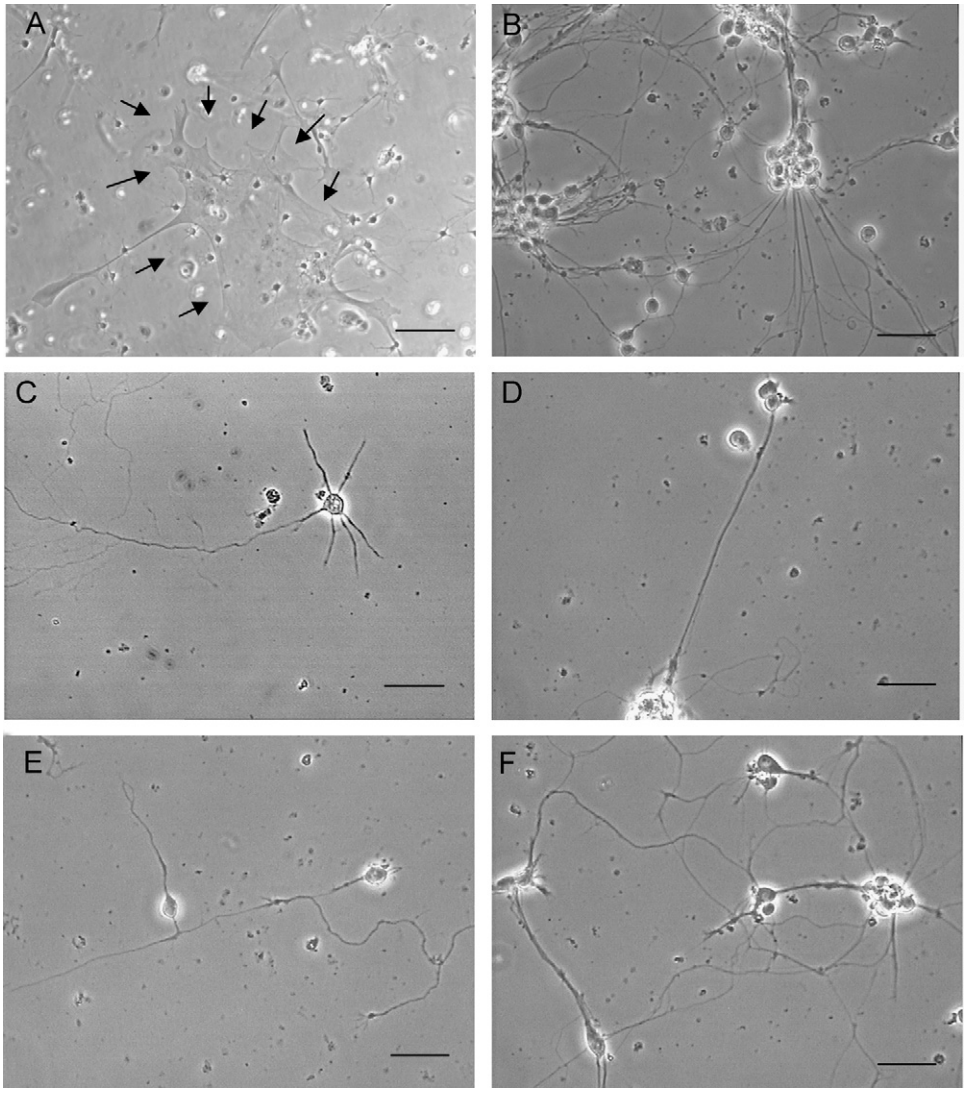

Fig. 1. (A) Carpet of glia grown in medium supplemented with horse serum (arrows indicate limits of the patch). (B) Glia-free cultures of frontal cortex neurons from postnatal rats (P1–P7) after 7 days in vitro aggregating in clusters connected by processes (B), in isolation (C), and forming simple networks (D, E and F). Scale bars 120 μm (A) and 60 μm (B–F).

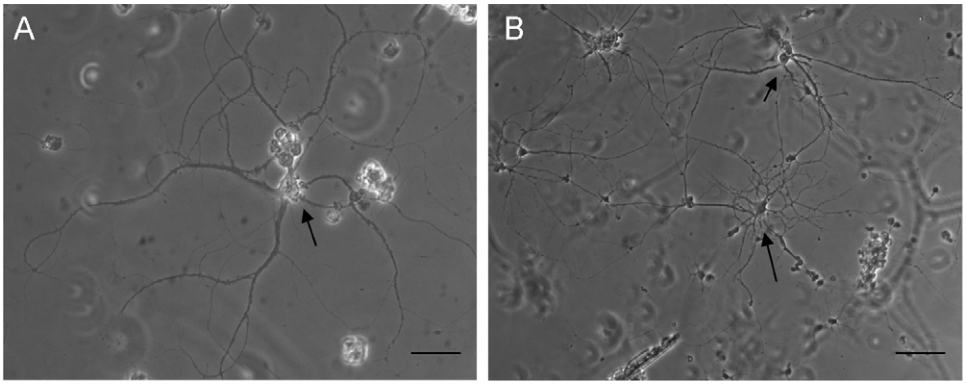

Fig. 2. In vitro neurons from postnatal rats (P1–P7) 4 weeks after plating, cultured at approximately 100 μm from a feeder layer of glia which provides the necessary factors for long-term survival while avoiding direct physical contact between the neurons and the glial carpet. Scale bars 40 μm (A) and 80 μm (B).

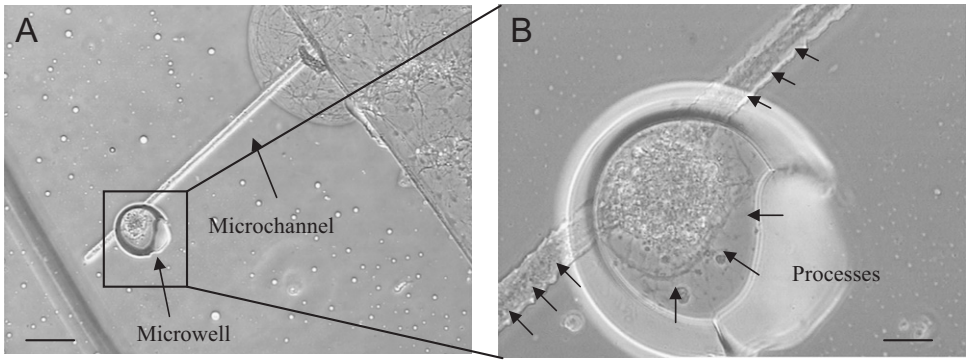

Fig. 3. Neuronal culture from rat frontal cortex 2 weeks after plating, patterned by a microstructured polymer film including a microwell and a microchannel (scale bars 120 and 20 μm in A and B, respectively).

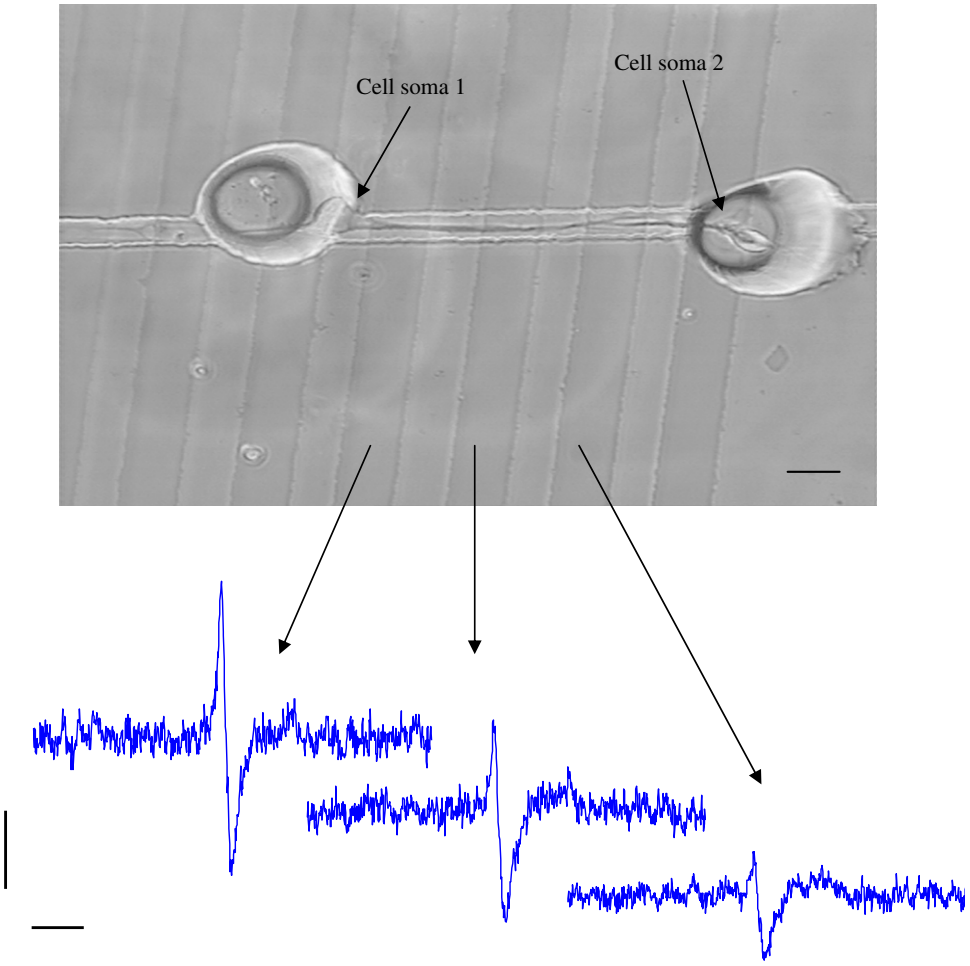

Fig. 4. Pair of connected snail neurons cultured within a PoM device (top) after 7 days in vitro and extracellular spikes recorded at three sites located in the lumen of the interconnecting channel (bottom). Scale bar 40 μm (top), 10 ms and 70 μm (bottom).

current–source density (CSD) analysis to interpret the recorded spikes and to assign them to individual neurons.

3.4. Spike-like and synaptic-like extracellular signals

An important goal of PoM devices, and of devices for interfacing with living neurons in general, is to achieve sufficient sensitivity to measure potentials of synaptic origin in addition to full spikes. The former correspond to small variations of membrane potential (~1 mV) with relatively long time constants (10–100 ms) produced by neurotransmitter release at synaptic connections. The later are associated with action potentials, i.e. changes of up to 100 mV in membrane voltage and duration of the order of 1 ms.

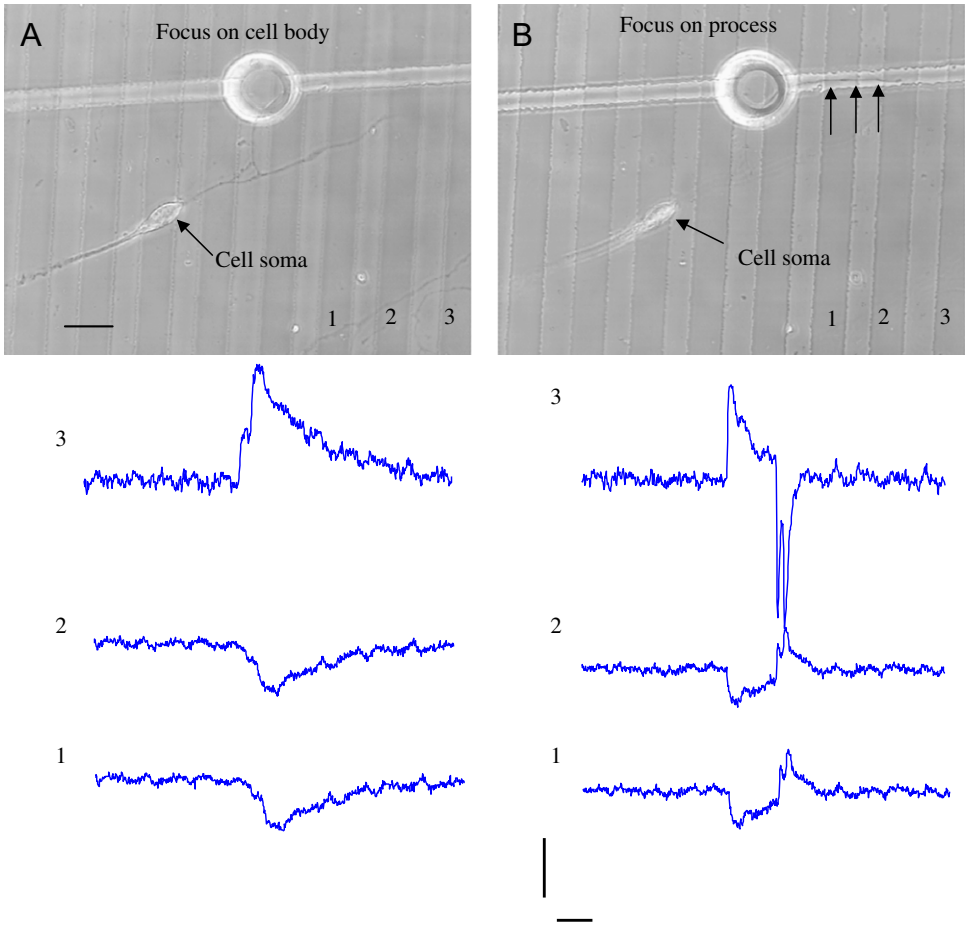

Fig. 5. Individual snail neuron displaced out of the microwell (A) and retaining a process segment within the microchannel (arrows in B). The bottom traces show two types of signals recorded by three electrodes (labelled 1–3). The left-most column contains a slow synaptic-like signal. The right-most column shows a similar signal truncated by a pair of spikes. Scale bars 60 μm (A and B), 200 μV and 10 ms (bottom traces).

Fig. 5A shows a cell soma on the polymer film with extensive neurite growth. Focusing on the substrate below the soma (Fig. 5B), the image shows a neurite segment remaining within the microwell and extending approximately 120 μm into the channel. In the course of the current investigations, several neurons grew out of the microwells, pulled mechanically by their neurites, and on occasions, despite migration of the cell body, a substantial neurite segment remains within the channel and produces measurable extracellular potentials. While displacement of the soma out of the well is not desirable in the context of PoM recordings, the example shown in Fig. 5 is particularly relevant because the signals at three sites within the channel (bottom traces) contained two electrical signatures with different profiles in time suggestive of both suprathreshold and synaptic (subthreshold) origins. The left-most column of traces shows spontaneously generated long time constant signals (in the order of tens of ms), while the right-most column contains similar signals terminated by fast spikes (approximately 1 ms). These data are consistent with synaptic potentials that do not produce an action potential in the left-most recordings but do so in the right-most traces.

If confirmed, for example by pharmacological abolishment of synaptic transmission, these results would confirm the capability of PoM devices to record low magnitude synaptic potentials.

3.5. *Future work*

Two main objectives will guide future work, namely, the development of PoM technology compatible with vertebrate neurons and the achievement of one-to-one electrical interaction with synaptically connected invertebrate neurons.

Towards the first goal, culture of individual vertebrate neurons within microwells with high survival rate and process growth is necessary and, accordingly, techniques for positioning the cells and promoting growth will be needed.

To realise interaction with invertebrate networks, the probability of growth and connection needs improvement and this will be explored by the use of growth factors as contained in conditioned medium. Further progress would result from systematic characterisation of the extracellular signals associated with sub-threshold (synaptic) activity.

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