Nanowire Array Technologies for Investigation of Neural Activity

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ABSTRACT

Nanostructures are expected to play a key role in the fundamental investigation of neuronal activity at the nanoscale level. Neurons are complex structures with feature sizes ranging from the millimeter to micron to nanoscale regimes. Single nanowires and/or nanowire arrays can therefore offer the potential to investigate the operations of living neural networks in precise detail, tracking small changes in cell structure and electrical activity in a minimally invasive fashion and at the same time with excellent spatial and temporal resolution. Such studies should thus improve our understanding of brain functions.

We report our results on the development of an array of conducting gold nanowires suitably integrated with gold electrodes for the recording of neural activity. Our preliminary results in the development of such a device and the interaction of such nanowire-arrays with cultured mouse neuroblastoma cells and rat hippocampal cells are discussed.

Keywords: Biorecognition, brain-machine interface, gold, nanoelectrode, nanowire array, neuronal network, peptide

1 INTRODUCTION

Nanotechnology, including nanoscale wire array-neuron interfaces, offers the opportunity to investigate the operations of neural systems with unprecedented detail. A better understanding of spatially complex processes such as neuronal integration, synaptic plasticity, memory and learning would have far-reaching implications. Moreover, nanowire devices have the potential of meaningfully integrating with neural tissue in a variety of clinical applications.

Currently, multi-electrode arrays (MEA) permit extracellular recordings as single unit activity or slow field potentials. However, these devices do not offer the level of resolution to record from smaller regions of a cell while still maintaining sufficient signal coherence. A gold nanowire interface, which is electrically conducting and biocompatible, can provide reliable multi-site electrical stimulation and recording capability for long-term monitoring of individual neurons. Additionally, using biorecognition molecules such as cyclic peptides, a direct interface between the nanowire and the neuronal cell membrane can be created to produce controlled cell growth on the surface of the nanowire array. The developing technology described here shows an example of how nanotechnology offers a unique range of opportunities to effectively record from neuronal cells.

2 Materials and Methods

2.1 *Fabrication of Au nanowires*

Electrochemical anodization and electrodeposition provides a feasible means by which to produce an array of gold nanowires inside a nanoporous alumina template. Therefore, we first prepared nanoporous alumina templates with controlled pore diameter and length which was attained by anodizing commercially available 99.995% pure aluminum foil in electrolytic solutions, such as 15% sulfuric acid, 3% oxalic acid, 5% phosphoric acid, etc. The anodization was carried out under *DC* conditions by placing an aluminum foil and a platinum mesh as the anode and cathode respectively. Maintaining a separation of 10cm produced a uniform electric field at the Al. Smaller pore diameters of 10nm were produced by anodizing Al foil with 15% sulfuric acid at 10V. To create templates with larger diameters of 40nm and 80nm, foil was anodized in 3% oxalic acid at 40V and 10% oxalic acid at 60V respectively (Fig 1.). Consistent results of 10nm pores were obtained by anodization with 15% sulfuric acid at 10V. Images for these dimensions have not been shown since it is difficult to capture pictures with suitable resolution.

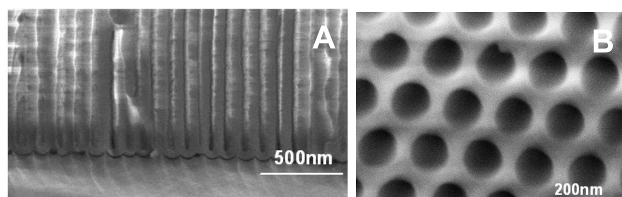

Fig. 1: SEM images showing pore patterns in nanoporous alumina. *(A)* Cross-section image showing vertically arranged pores with diameter 40nm prepared by anodization in 3% oxalic acid at 40V and *(B)* top view image of a template prepared by anodization in 5% phosphoric acid with pores diameter of 75nm.

To synthesize the gold wires within the template, electrodeposition was performed by using an electrolyte containing $HAuCl_4 \cdot 4H_2O$ (0.93 g/l) and boric acid, H_3BO_3 (30 g/l) with pH 1.5. Under *AC* conditions at 250Hz, 12V electrodeposition was achieved by controlled accumulation

of Au^{3+} ions, which combine with 3 electrons during every negative half cycle. The length of gold nanowires described in this chemical reaction is determined by the electrodeposition conditions (time of deposition and *AC* frequency). The diameter of the wires reflects the original pore diameter of the template. Therefore, this method renders precisely controlled conditions allowing us to consistently replicate each gold nanowire array (Fig. 2).

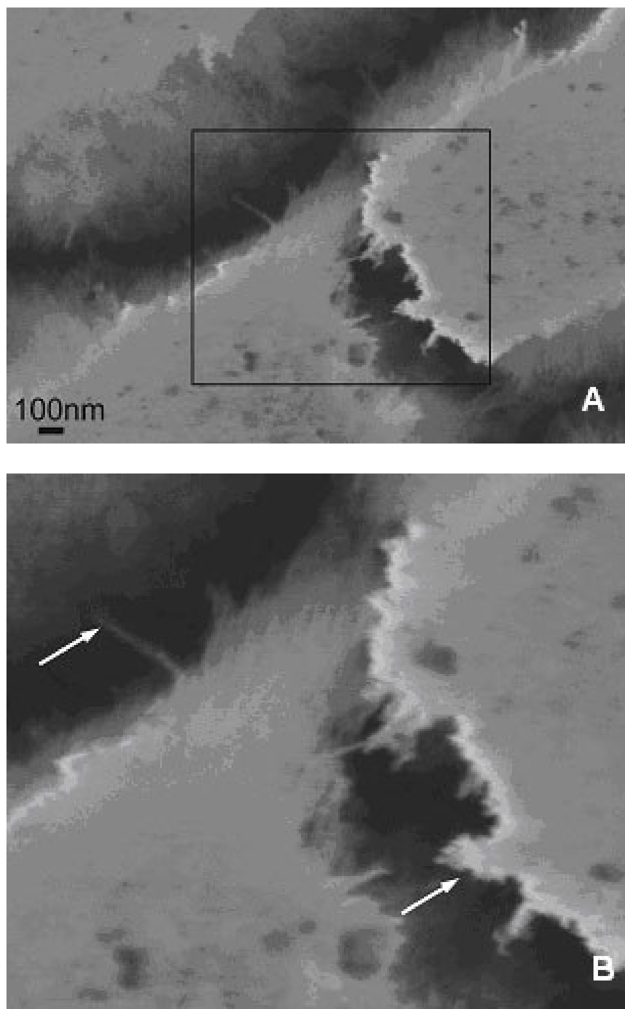

Fig. 2: SEM images showing the formation of gold nanowires. *(A)* Area of a nanowire array with the box magnified in *(B)* top arrow indicates a single gold nanowire and the lower arrow indicates many wires clustered together in bundles.

Next follows a series of steps to etch out the nanowires from both sides of the template. This begins by chemical etching and ion milling of the top surface of the aluminum oxide. The process begins with a chemical etching of the sample with a dilute (5%) phosphoric acid and is followed by ion milling to create a uniform length nanowire surface close to the template. The etched sample is then covered with a protective organic layer followed by removal of the aluminum layer in mercuric chloride solution. This is followed by etching of the thin barrier layer of aluminum oxide (~10-40nm) below the pores with dilute (5%) phosphoric acid solution. In the final step of this process, the protective organic layer is removed in alcohol. The resulting sample then consists of a protruding array of gold nanowires from both surfaces of the template where the midsection of the nanowires are firmly embedded inside the template.

To then integrate this nanowire array with a gold electrode array we begin by uniformly coating a thin layer of negative resist polymethylmethacrylate (PMMA) ~150nm at the surface of the template and baking at 180°C for 90 seconds for curing. Using CAD (computer-aided design) an appropriate resolution design was then patterned onto the PMMA in the scanning electron microscope (SEM) by E-beam lithography (EBL). A typical 2x2 electrode array with an area size of ~100μm^2 was prepared in this manner. The pattern was developed and the exposed region was removed in methyl-iso-butyl-ketone and iso-propyl alcohol solution. To assure specific gold adhesion, a final plasma etch is performed to remove any further traces of PMMA. The sample is then sputtered with gold and followed by a four day lift-off in acetone leaving gold microelectrodes attached to nanowires. The wire-bonded gold wire can then be attached to macroscopic wires in order to connect to external devices for recording and stimulation of neuronal cells (Fig. 3).

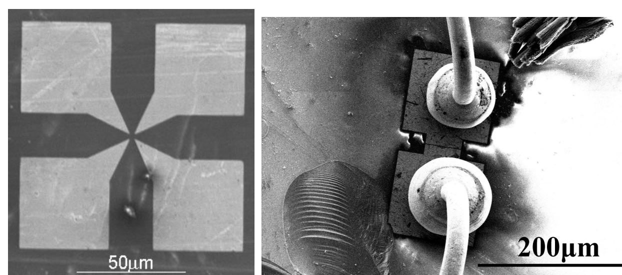

Fig 3: (left) A rectangular microelectrode array fabricated using e-beam lithography. (right) SEM image showing macroscopic wire bonding to electrodes.

2.2 *Peptide Conjugation*

The exposed gold nanowires on the top surface of the template provides an appropriate extension, which can then be functionalized with specific cyclic peptide sequences that target the integrin receptors on the extracellular matrix (ECM) of a cell. Since gold has been shown to have a strong affinity for thiol groups [1,2] to form strong bonds, peptide sequences with a free thiol group can then be directly attached to the nanowire surface. The binding affinity between cyclic peptides and the $\alpha\beta$ integrin subunits on the cell surface has been exploited in various modes of biomedical research [3]. The arginine–glycine–aspartic acid (RGD) peptide sequence is a commonly recognized motif by integrins where the arginine and aspartic acid constitute the active complex in most RGD molecular recognitions [4]. The peptide sequence selected for this study is CGGGRGDS, which will be anchored to

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gold through the free thiol group on the cysteine residue and the RGDS portion of the molecule will bind to the integrin complex on the surface of the cell.

For this study exposed gold nanowire templates and glass coverslips sputtered with 10nm of gold were used as the experimental and control studies respectively. In order to produce an efficient peptide coating on the exposed gold nanowires an appropriate concentration was determined. This can be modified further for greater stringency if a competitive thiol is introduced into the reaction. Each surface was cleaned in sterile deionized water and allowed to air dry. The 5mg peptide sequence, cyclo Arg-Gly-Asp-D-Phe-Cys (c(RGDfC); Peptides International, Inc.) was dissolved in 500μl 3% acetic acid. Then 10μl of the dissolved peptides was added to 990μl of 2.7μmol of HEPES buffer, pH 6.5. The buffered solution was then placed on the gold nanowires and the coverslips to incubate overnight (~18 hrs.). In final preparation for cell seeding, the samples were rinsed with PBS buffer.

2.3 *Neuroblastoma Cell Cultures*

As a means to evaluate the performance of gold nanowire arrays we began by culturing mouse neuroblastoma cells (N1E-115) purchased from ATCC on the opposite side of the EBL contact pads. The cells were cultured in flasks containing 90% Dulbecco's modified Eagles' medium with 4.5g/L glucose (without sodium pyruvate) (DMEM; Invitrogen), 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin and maintained at 37°C with 5% CO_2 in a humidified atmosphere and passaged every 3-4 days. These cells are a clone from the mouse neuroblastoma line C-1300 and retain many of the properties of differentiated neuronal cells. In order to create differentiated cultures, the cells were first plated on 30mm plastic dishes with 20mm glass inserts that were coated with 0.1% W/V poly-L-Lysine (Sigma) or 2.5 μg/ml ECL matrix (entactin-collagen-IV-laminin) (Upstate Cell Signaling Solutions) and incubated overnight at 37°C. The cells were then plated in differentiation medium (96% DMEM supplemented with 2.5% of FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin and 1.5% of DMSO) or serum starved. Medium was changed every 48hrs.

2.4 *Hippocampal Cell Cultures*

In order to better understand the patterning of synaptic connectivity between neurons at the level of individual synapses our study has now come to focus on the properties of hippocampal neurons. Therefore, neuronal hippocampal cultures were prepared from E18 Sprague-Dawley rats acquired from BrainBits[tm]. Dissociation of cells was performed by triturating the tissue through a fire-constricted Pasteur pipette and spun at 11000rpm for 1min. The supernatant was then discarded and the pellet was resuspended in B27/Neurobasal + 0.5mM glutamine without glutamate (Invitrogen) and plated onto enactin-collagen-IV-laminin (Upstate Cell Signaling Solutions) coated coverslips (12mm diameter) as described previously. The cultures were untouched for 3 days and then one half the media was changed every three days following. The cells were grown in a 37°C with 5% CO_2 and 20% O_2 in a humidified atmosphere.

3 RESULTS AND DISCUSSION

The human brain is a complex network of neurons with reciprocal connections through which information is transmitted and received. In studying the activity at synapses, which are the specialized contact zones between nerve cells, we may begin to understand the information processing capabilities of both single nerve cells and systems of nerve cells. Therefore, the development of nanowire devices should advance our understanding of the specific patterns of neural activity associated with learning and memory are created as well as the mechanisms of plasticity within neuronal networks.

To study complex networks, a vertically arranged nanoelectrode array that has the capability to stimulate and record from several different sites on a neuron would be quite useful. Applying this to cultured networks, in which neurons are in direct contact with the electrode arrays, should reveal network level flows of information that underlie neural computations including sensory information processing, memory storage and motor programming.

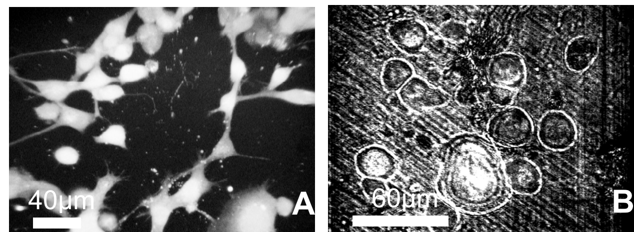

Fig 4: Mouse neuroblastoma cells in culture. *(A)* 5 day N1E-115 cells cultures labeled with Fluo-4 and visualized by confocal microscopy. *(B)* 3 day N1E-115 cells cultured on a gold nanowire array. The cross-grain of the template can been seen traversing at a diagonal.

Our preliminary experiments of neuroblastoma cell cultures on the gold nanowire surface has shown that while the cells were viable, the interface between the exposed nanowires and the cell matrix was rather poor, resulting in loosely aggregated groups of cells sprawled on the array (Fig. 4).

In order to create the cellular conditions that are required of primary neuronal cultures, we began to grow primary cultures from 18 day old embryonic rat tissue. As seen in figure 5, active networks are formed spontaneously from dissociated cultures by extending axonal branches and dendritic processes. Confocal calcium imaging was used to functionally assess these hippocampal cultures and showed that they are widely electrically excitable. The robust calcium responses observed will provide a powerful means to evaluate the spatial and temporal capabilities of the

nanowire arrays—in both neural stimulation experiments and during recordings from intricate neural networks.

To improve the cell viability and the adherence of these primary cultures on the gold nanowire arrays, cyclic peptides were attached to the tips of the exposed nanowires. The attachment of such biorecognition molecules to gold nanoparticles has become a standardized method and a wide variety of linker molecules with various functional groups and varying lengths are available [5]. One of the most commonly used approaches to bind biomoleculaes to gold is through thiol bond formation, which creates an irreversible bond. Therefore our coupling method uses the thiol bond on the cysteine containing peptide to conjugate to the gold. Figure 5 shows the conjugation of the biorecognition molecule to the gold nanowire arrays with improved cell viability, though cell adhesion to the gold nanowire surface still remains a challenge. Nonetheless, there is a marked improvement over the previous results shown with the mouse neuroblastoma cells, where there was very limited cell survival (Fig. 4). Furthermore, the development of the gold nanowire array promises utility in investigating live neural networks with far greater special and temporal resolution than is presently possible.

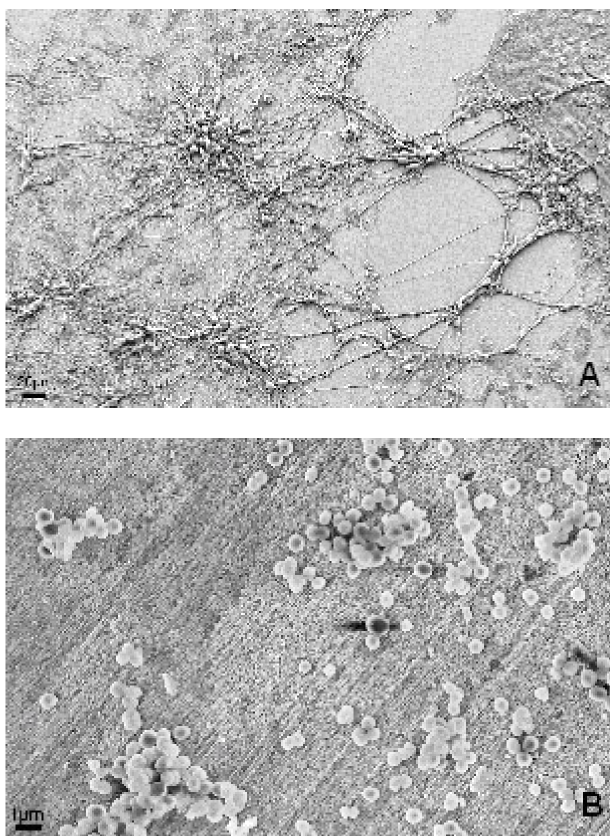

Fig 5: E(18) hippocampal culture morphology. *(A)* 5 day primary cultures of hippocampal cells imaged by SEM. *(B)* 5 day old hippocampal cells on a gold nanowire array conjugated to biorecognition molecules. SEM image shows the cross-grain morphology of the template.

Over the long-term, this type of instrument can be used to study neuroplasticity, axon guidance during development, and distinct modes of learning and memory. Such arrays can also provide a high bandwidth interface to the nervous system to enable the precise control of implants and prosthetic devices.

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