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Biocompatible Parylene Neurocages

Developing A Robust Method for Live Neural Network Studies

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We present a refined method and design for fabricating parylene neurocages for in vitro studies of live neural networks. Parylene neurocages are biocompatible and very robust, making them ideally suited for studying the synaptic connections between individual neurons to gain insight into learning and memory. The neurocage fabrication process is significantly less complex than earlier versions. Previous neurocage designs achieved limited neuronal outgrowth; however, the long-term cell survival rate was <25%. As outlined here, the incorporation of new materials and different anchoring techniques, in addition to some design modifications, have improved the long-term cell survival rate to >50%.

Neurons play an important role in many of our biological and cognitive functions. Many studies concentrate on the properties of neurons and the neural networks they form; unfortunately, it is difficult to study these networks in vivo. Initial in vitro techniques used patterned extracellular electrode arrays [1], [2], but neuron mobility and lack of neuron-to-electrode specificity limit the use of these arrays, especially in long-term studies.

Our strategy counteracts this difficulty by using micromachined structures to physically trap individual neurons in close proximity to electrodes without inhibiting their growth. The first implementation was the neurowell [3], [4]. This concept involved etching wells in bulk silicon and then adding a nitride canopy to cover the top. The canopy contained openings to allow the outgrowth of neurites, while at the same time trapping a neuron in close proximity to an electrode (Figure 1). Arrays of neurowells permitted the neurites from different neurons to form connections, thereby allowing the development of neural networks. With these neurowells, individual neurons in live neural networks could be reliably stimulated and recorded from for long-term studies. While greatly aiding the study of live neural networks, the fabrication and scaling complexities of the neurowells limited their continued development. In addition, the neurons in the neurowells tended to be pulled away from the bottom of the well, and hence from the electrode, by the neurites growing out through the channels on top of the well.

To address these problems, our group developed surface micromachined parylene neurocages [5], [6]. Unlike in the neurowells, neurites grow out the bottom of the neurocages

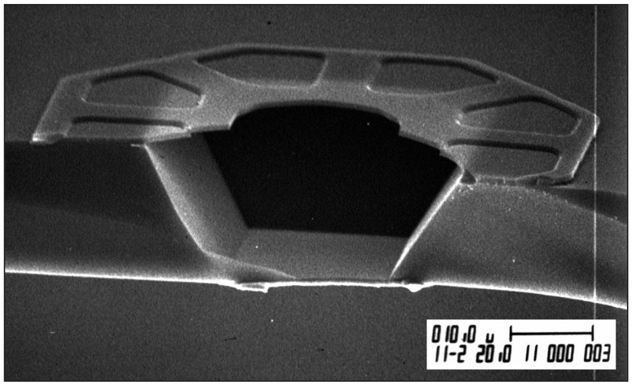

Fig. 1. SEM of neuro-well cross section.

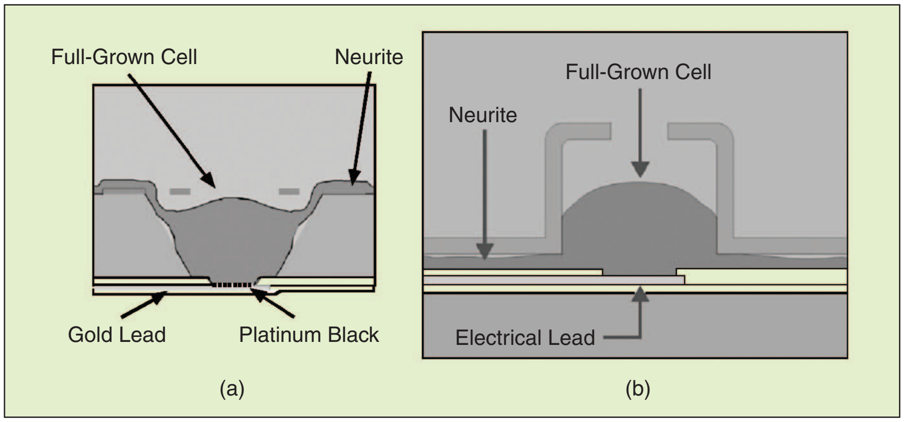

Fig. 2. (a) The neuron loaded in the neuro-well and (b) the neurocage.

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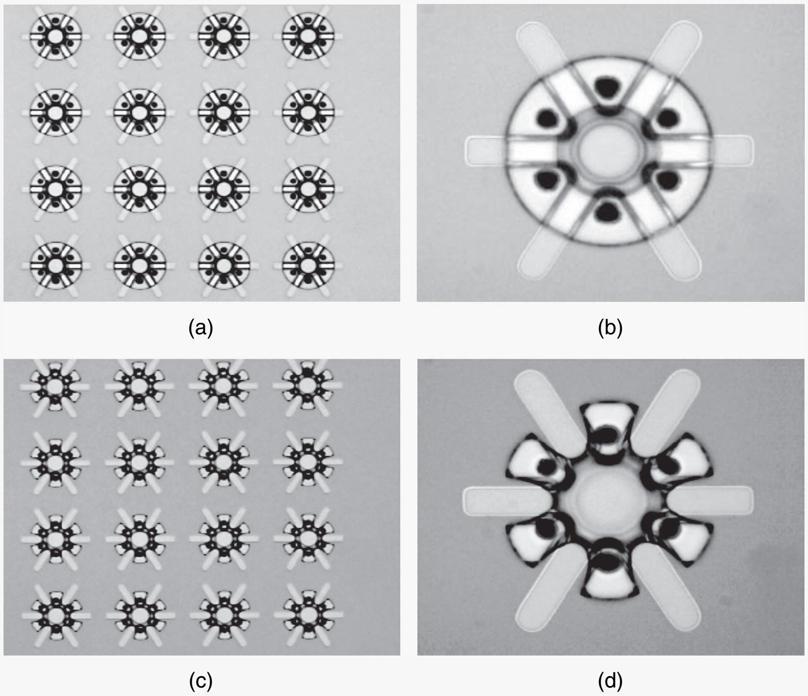

Fig. 3. SEMs showing top views of (a) 4 × 4 array of neurocages with 40-μm tunnel lengths and 10-μm tunnel widths; (b) a neurocage with 40-μm tunnel lengths and 10-μm tunnel widths; (c) 4 × 4 array of neurocages with 4-μm tunnel lengths and 10-μm tunnel widths; and (d) a neurocage with 4-μm tunnel lengths and 10-μm tunnel widths.

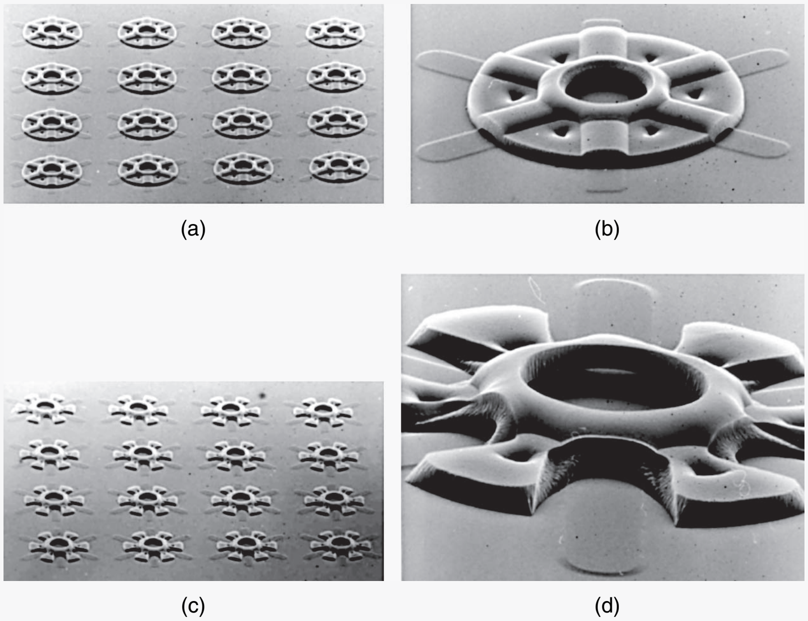

Fig. 4. SEMs showing tilted views of: (a) SEM of a 4 × 4 array of neurocages with 40-μm tunnel lengths and 10-μm tunnel widths; (b) an SEM of a neurocage with 40-μm tunnel lengths and 10-μm tunnel widths; (c) an SEM of a 4 × 4 array of neurocages with 4-μm tunnel lengths and 10-μm tunnel widths; and (d) an SEM of a neurocage with 4-μm tunnel lengths and 10-μm tunnel widths.

(Figure 2), pulling the neuron closer to the electrode.

Parylene was chosen to be the structural material in this application because it is biocompatible, nontoxic, extremely inert, and resistant to moisture and most chemicals. Hence, parylene is well suited for long-term cell culture experiments. Its conformal deposition makes it easy to fabricate three-dimensional structures like the neurocage. In addition, parylene is transparent; thus, when neurons are loaded into neurocages, they can easily be seen.

The initial neurocage design achieved some neuron outgrowth, but long-term cell survival was low (<25%). The new neurocage process and design presented here, while preserving several elements of the previous designs, increases the long-term cell survival rate to >50%.

Methodology

Design

The neurocage consists of a chimney 30 μm in diameter and 4 μm high with a 15-μm-diameter inlet hole at the top for loading neurons. Extending out from the chimney are six tunnels for neuron outgrowth interleaved with six anchors for mechanical stability. The tunnels are 1.5 μm high and either 5 μm or 10 μm wide. They extend for either 40 μm or 4 μm (the thickness of the deposited parylene, effectively creating a slot in the side of the chimney rather than a tunnel). The neurocage array (4 × 4) consists of 16 neurocages, each designed to hold a single neuron, centered within a 440 μm × 440 μm square (Figures 3 and 4).

Fabrication

The process flow for creating the neurocages is shown in Figure 5. First, a thin layer of oxide, approximately 500 nm thick, is grown on a silicon substrate. The anchors for the parylene neurocage are then patterned, and the oxide is etched using buffered oxide etch (BHF).

A partial exposure method uses two separate exposures with different masks to define the chimneys and tunnels using only a single layer of photoresist (AZ4400). After developing, both of these features are created.

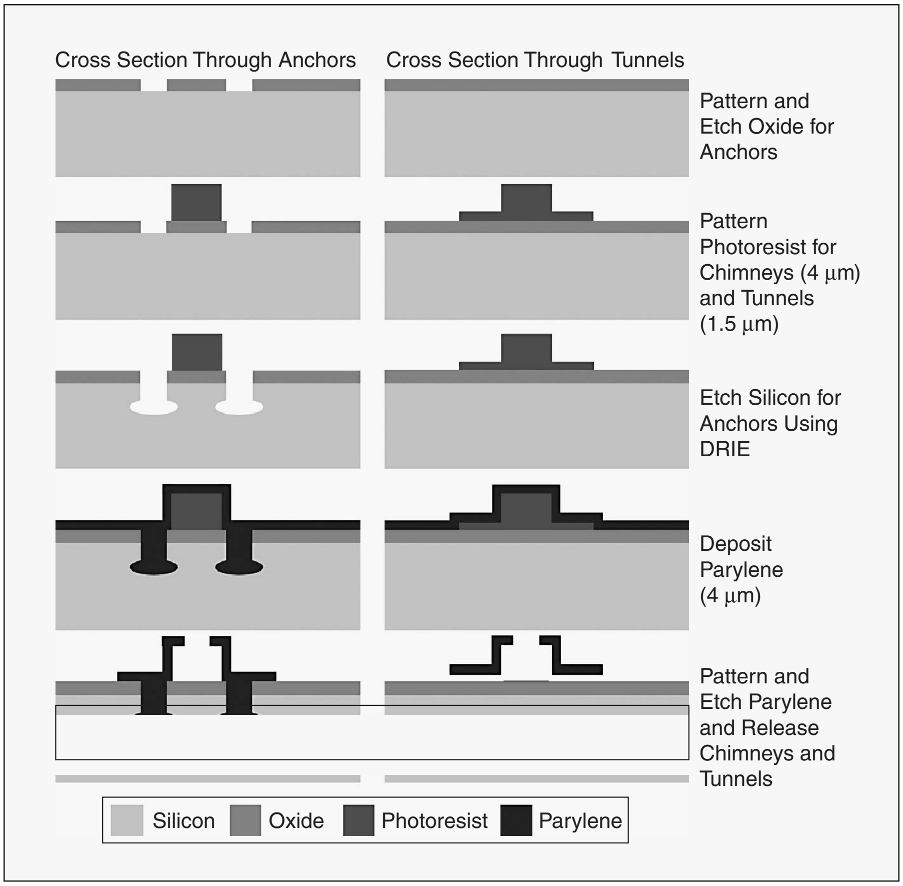

Fig. 5. The fabrication process flow.

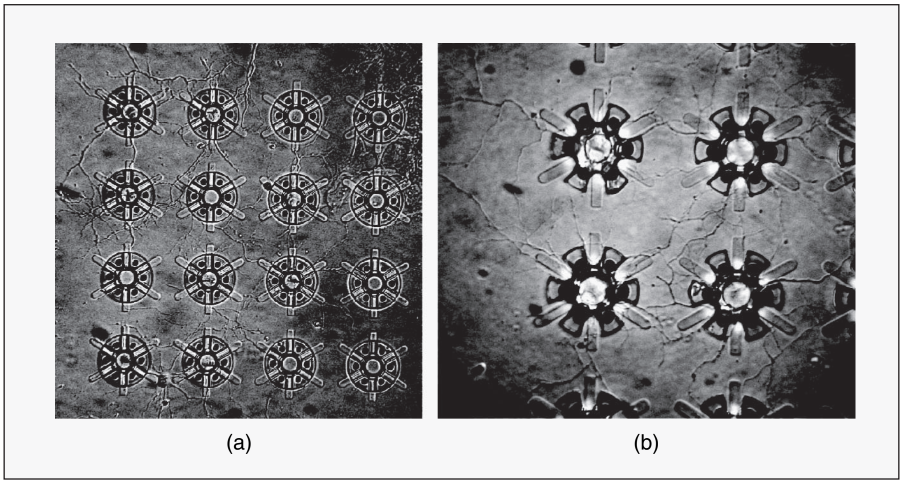
(a) (b)

Fig. 6. (a) Nomarski picture of neurons and neurite outgrowth inside the neurocages with 40-μm tunnel lengths and 10-μm tunnel widths. (b) Nomarski picture of neurons and neurite outgrowth inside neurocages with 4-μm tunnel lengths and 10-μm tunnel widths.

The anchors are then etched into the silicon using a deep reactive iron etch (DRIE) process developed in our group for mechanically securing parylene to a substrate [7]. The DRIE uses a modified Bosch process: 50 loops of a standard Bosch process to make an anisotropic trench with nearly vertical sidewalls and a subsequent 30-s SF_6 isotropic etch to create a mushroomlike bottom. The anchors are 10–50 μm deep.

Subsequently, a single layer of parylene is deposited then patterned and etched using O_2 plasma to create the neurocages. The previous fabrication process required two depositions of parylene. The sacrificial photoresist defining the chimneys and tunnels are released using acetone. Finally, the neurocages are cleaned using piranha (5:1:1 H_2SO_4:H_2O_2:H_2O) at 120 °C for 10 min followed by a 10-s hydrofluoric acid (HF) dip.

Cell Culture

After sterilization with UV light, the neurocages are covered with 95% EtOH. The EtOH is then exchanged for water. Five percent poly-ethylene-imine (PEI) is added to promote cell adhesion to the substrate. PEI is rinsed out of the dish and subsequently exchanged for neurobasal medium. Neurons are then plated at a density of 30,000 K/cm^2. Cells are loaded manually into the neurocages with a pressure-driven micropipette. The first signs of neuron growth usually appear within 12–24 h of loading.

Results

Neurocages produced using this new fabrication process are mechanically robust and able to withstand various cleaning procedures, including acetone and piranha, with no deformation or delamination. In addition, initial studies have shown that neurocages can survive for long periods of up to 80 days in saline at 30 °C with no visible deformation or delamination. (The study was concluded after 80 days; therefore, no data is available for longer periods.) Based on these studies, the neurocages should suffer no adverse effects when placed in the neurobasal medium for long periods.

Successful growth of live neural networks has been achieved using 4 × 4 arrays of neurocages (Figure 6), thereby proving that the neurocages are biocompatible. Neuron outgrowth has been achieved in neurocages with tunnel widths of both 5 μm and 10 μm and lengths of either 40 μm or 4 μm. No significant differences in neuronal survival rate and outgrowth have been noted due to the different combinations of tunnel lengths and widths.

In addition, these neurocages can be cleaned of all neuron debris using piranha and HF for reuse in growing live neural networks (Figure 7).

Discussion

The current neurocage design and process, while similar to previous versions, dramatically increases the long-term cell survival rate. In previous designs, the chimney was 15 μm high, the tunnel heights varied from 0.3–2 μm (depending on the fabrication process used), and the tunnel length was 30 μm. In the current design, the chimney height is 4 μm, the tunnel height is 1.5 μm, and the tunnel lengths are 4 μm and 40 μm. Clearly, the biggest difference between the current design and previous designs is the chimney height. The reduced chimney height in the current design seems to be a primary cause for the increased survival rate.

Another potential cause for the increased cell survival rate is the fabrication process. Previous neurocage designs used two separate lithography processes to build the tunnels and chimneys. These tunnels were formed by sputtered silicon, hardbaked photoresist, or thermally evaporated aluminum, while a thick layer of photoresist (AZ9260) formed the chimneys. As a result, more drastic release methods were required: BrF_3 or XeF_2 gas etching for the sputtered silicon, ST-22 photoresist stripper for the hardbaked photoresist, or Al etchant for the thermally evaporated aluminum. With these methods, it was not always possible to ensure that the materials used to form the tunnels had been completely removed. If any of this material remained, it could block the tunnel, thus preventing neuronal outgrowth, or, as in the case of the hardbaked photoresist, it could kill the neurons (photoresist is toxic to neurons). With the current fabrication process, the tunnels can be released using acetone, and it is easier to make certain that all photoresist has been removed.

Although impoved long-term cell survival is achieved with the new neurocage design, it is not clear whether the success is attributed to the reduced chimney height or to the fabrication process. To definitively answer this question, it would be necessary to fabricate neurocages with reduced chimney height using the previous process flow. (Limitations of the partial exposure method prevent it from being used with 15-μm-high chimneys.)

Previous designs used BrF_3 or XeF_2 to etch the anchors. With these methods, however, it was not possible to accurately control the undercut associated with the isotropic nature of the etching process. The undercut caused the size of the anchors to increase, thereby shrinking the area available for the tunnels, and in some cases, eliminating the tunnels. With the DRIE process used for the current neurocages, the undercut can be reliably controlled.

In the current process, the anchors are not etched into the silicon as part of the initial step because the subsequent lithography step, to create the tunnels and chimneys, allows photoresist to flow into the anchors. Since the anchors cover such a small surface area and are comparatively deep, it is not possible to ensure that the photoresist is completely removed from the anchors during the development process. Remnants of photoresist left in the anchors counteract the ability of the anchors to firmly secure the parylene neurocages to the surface, often causing them to release during subsequent cleaning procedures.

Conclusions

The design and process presented here for parylene neurocages can be used for in vitro studies of live neural networks. This fabrication process is less complex than previous neurocage and neurowell fabrication processes. Biocompatible and robust neurocages can be created that achieve significantly higher neuronal survival and outgrowth rate than previous versions. The next step is to incorporate platinized gold electrodes into the neurocages to stimulate and record from individual and groups of neurons.

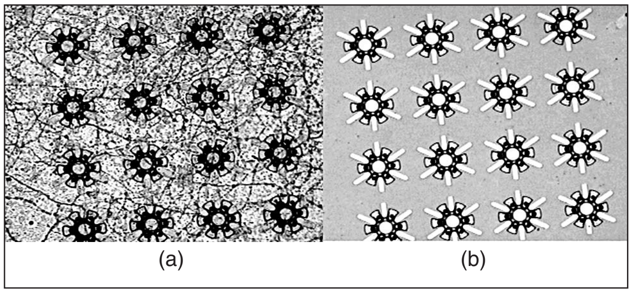

Fig. 7. (a) A neurocage array after neural network growth. Note the debris in and around the cages. (b) A neurocage array after cleaning in 10 min piranha, followed by 15-s HF dip.

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