

DEVELOPMENT OF BIOCOMPATIBLE PARYLENE NEUROCAGES

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Abstract—We present a refined method and design for building 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%. The incorporation of new materials and different anchoring techniques, in addition to some design modifications, as outlined here, have improved the long-term cell survival rate to >50%.

Keywords—neurocage, neuron, neuro-well, parylene

I. INTRODUCTION

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 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 neuro-well [3,4]. This concept involved etching wells in bulk silicon,

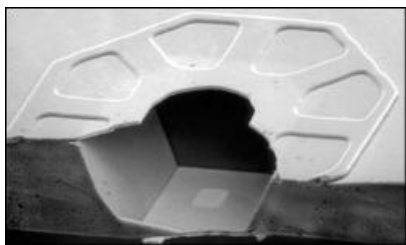


Fig. 1. SEM of a neuro-well.

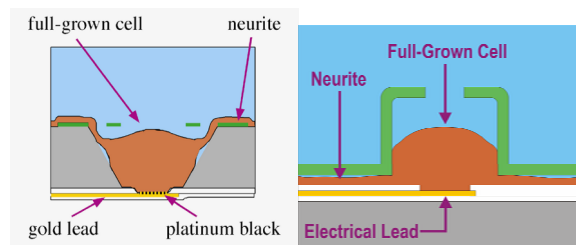


Fig. 2. Neuron loaded in neuro-well (left) and neurocage (right).

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 (Fig. 1). Arrays of neuro-wells permitted the neurites from different neurons to form connections, thereby, allowing the neurons to develop into neural networks. With these neuro-wells, 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 neuro-wells limited their continued development. In addition, the neurons in the neuro-wells 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 neuro-wells, neurites grow out the bottom of the neurocages (Fig. 2), pulling the neuron closer to the electrode.

Parylene was chosen to be the structural material in this application because it is biocompatible, non-toxic, 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%.

II. METHODOLOGY

A. Design

The neurocage consists of a chimney, 30 μ m in diameter and 4 μ m high, with a 15 μ m inlet hole at the top for loading neurons (Fig. 3). Extending out from the chimney are 6 tunnels, for neuron outgrowth, interleaved with 6 anchors, for mechanical stability. The tunnels are 1.5 μ m high and either 5 μ m or 10 μ m wide. They extend for either 40 μ m or 4 μ m (the thickness of the deposited parylene, effectively creating a slot in the side of the chimney rather than a tunnel). The neurocage array (4 x 4) consists of 16 neurocages, each designed to hold a single neuron, centered within a 440 μ m x 440 μ m square (Figs. 4-5).

B. Fabrication

The process flow for creating the neurocages is shown in Fig. 6. First, a thin layer of oxide, approximately 500nm thick, is grown on a silicon substrate. The anchors for the parylene neurocage are then patterned, and the oxide is etched using BHF.

The 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, these features are created.

The anchors are then etched into the silicon using a 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 second SF₆ isotropic etch to create a mushroom-like bottom. The anchors are 10 μ m-50 μ m deep.

Subsequently, a single layer of parylene is deposited, covering the whole surface, which is then patterned and

etched using O₂ 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 H₂SO₄:H₂O₂:H₂O) at 120°C for 10 minutes followed by a 10 second HF dip.

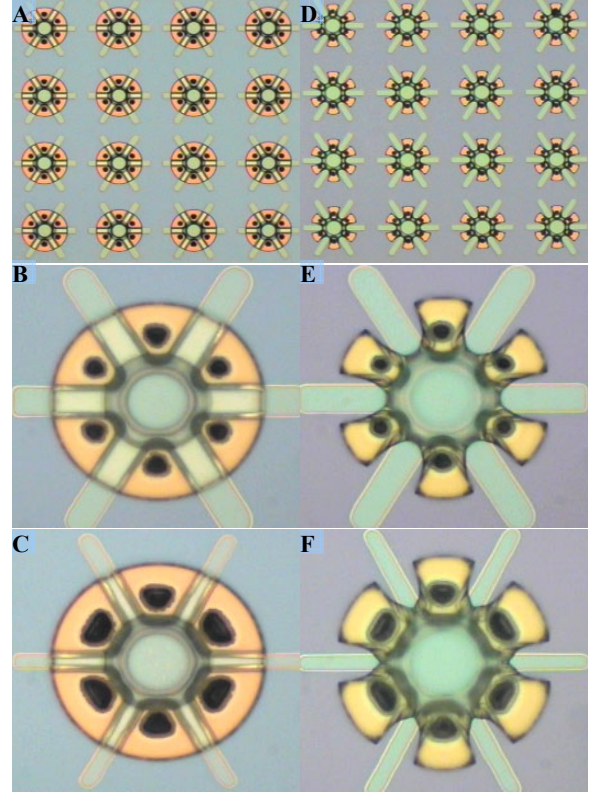


Fig 4. Optical Pictures of Neurocages (A-C) 40 μ m tunnel length. (A) 4x4 Array, 10 μ m tunnel width. (B) 10 μ m tunnel width. (C) 5 μ m tunnel width. (D-F) 4 μ m tunnel length. (D) 4x4 Array, 10 μ m tunnel width. (E) 10 μ m tunnel width. (F) 5 μ m tunnel width.

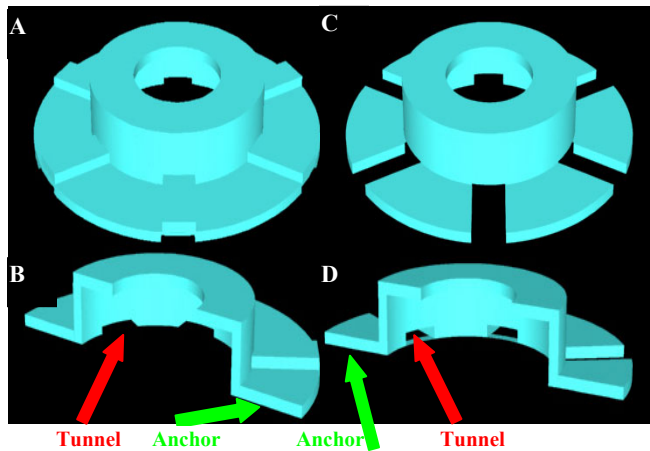


Fig 3. 3D Neurocage Model (A) 40 μ m tunnels. (B) Cross-Section with 40 μ m tunnels. (C) 4 μ m tunnels. (D) Cross-Section with 4 μ m tunnels.

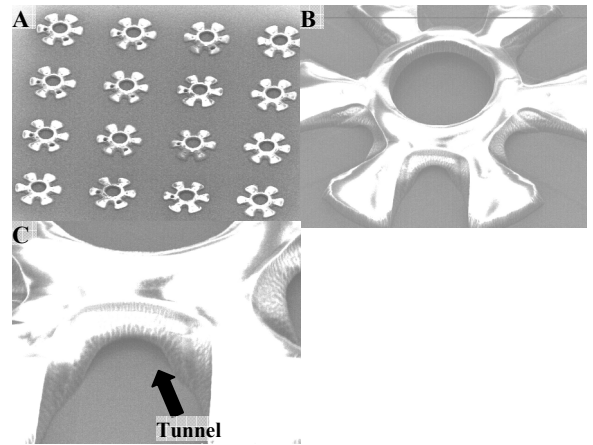


Fig 5. SEM Pictures of Neurocages, 4 μ m tunnel length. (A) 4x4 Array, 5 μ m tunnel width. (B) 10 μ m tunnel width. (C) 10 μ m tunnel width.

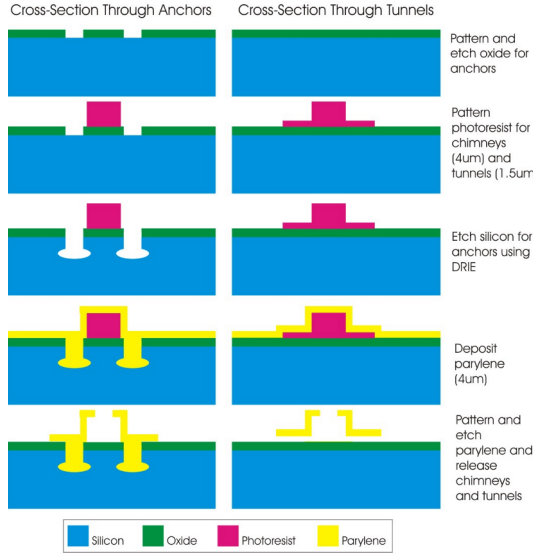


Fig 6. Process Flow.

C. Cell Culture

After sterilization with UV light, the neurocages are covered with 95% EtOH. The EtOH is then exchanged for water. 5% PEI (poly-ethylene-imine) 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 30K/cm². Cells are loaded manually into the neurocages with a pressure-driven micropipette. The first signs of neuron growth usually appear within 12-24 hours of loading.

III. RESULTS

Neurocages produced, using this new fabrication process, are mechanically robust, 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, up to 80 days, in saline at 30°C with no visible deformation or delamination. (The study was concluded after 80 days; hence, 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 x 4 arrays of neurocages (Fig. 7), thereby proving that the neurocages are biocompatible. Neuron outgrowth has been achieved in neurocages with tunnel widths of both 5μm and 10μm, and lengths of either 40μm or 4μm. 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 re-use in growing live neural networks (Fig. 8)

IV. 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 15μm high, the tunnel heights varied from 0.3μm to 2μm (depending upon the fabrication process used), and the tunnel length was 30μm. In the current design, the chimney height is 4μm, the tunnel height is 1.5μm, and the tunnel lengths are 4μm and 40μm. 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

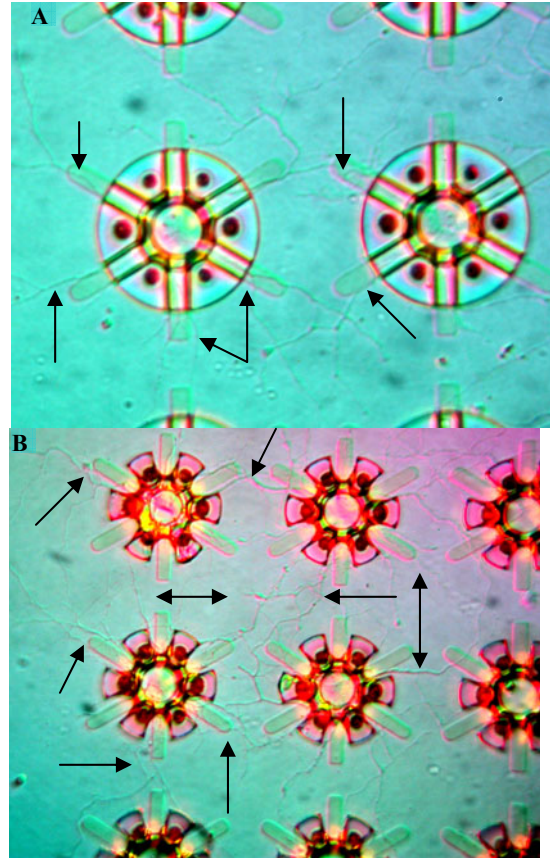


Fig 7. Nomarski Pictures of neurons inside neurocages, with neurite outgrowth. (A) 40μm tunnel length, 10μm tunnel width. (B) 4μm tunnel length, 10μm tunnel width. Arrows denote neurite outgrowth.

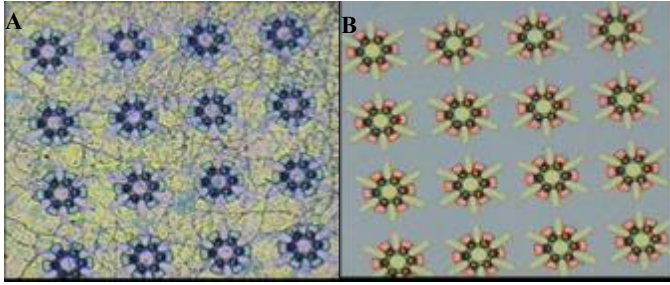


Fig 7. (A) Neurocage array after neural network growth. Note debris in and around the cages. (B) Neurocage array after cleaning (10 minutes piranha, followed by 15 second HF dip).

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 good long-term cell survival can be achieved with the new neurocage design, it is not clear whether the success can be attributed to the reduced chimney height or to the fabrication process. To definitively answer this question, it would be necessary to use the previous fabrication processes with the reduced chimney height. (Limitations of the partial exposure method prevent it from being used with $15\mu\text{m}$ 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. 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.

V. CONCLUSION

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 neuro-well 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 neurons.

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