BIO-MEMS DEVICES TO MONITOR NEURAL ELECTRICAL CIRCUITRY

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Many approaches are possible when considering the combination of microelectronics and nerve cell circuits, though before this science fiction can become a reality the knowledge regarding the nerve cells and their interactions must approach that of the rest of the system. The task set before the engineers involves many factors which span different engineering disciplines and are too broad to be covered in full with this paper. Among these concerns are signal processing, neuron-microelectronic interface, and support of the entire system. At this point, not enough is known about the organic side of these hybrid circuits so research has been moving to remedy this. The focus primarily moves to the interface, and later, the knowledge and methods gained and developed can be used to shed light on signal processing within natural occurring neural networks; either regarding the basic knowledge of signal transmissions within normal neurons or how to descramble those signals when it has been passed to the rest of the circuit. A method had to be developed where individual neurons could be tested and retested under complete control of the researchers. This allows the researchers to get an accurate portrayal of the signals transmitted and the effect of outside influences on those transmissions. In this report, we include three different ways to achieve such goal for discussion. They are planar culturing system, small neuron network by cages on chip and living neuron chip with peptide amphiphiles gels.
Part I: Planar culturing system [1,2]

The original solution was a planar array of stimulator-transistor clusters. These interfaces allowed the researchers (Jenkner M, Müller B, Fromherz P, 2000) to place and subsequently stimulate neurons or pairs of neurons. This was an elementary step in the direction of any number of other more sophisticated projects. By creating the silicon-neuron junction for capacitive stimulation and recording, it was now possible to study the response of the neural “network” without damaging the cell with the more invasive technique of microelectrode piercing. While more precise and clean, the signal recorded from the microelectrode meant that the cell only remained alive for a few hours, meaning that the opportunity for retesting of the same neuron cell or cell network was severely limited. Using the capacitive method, the neuron and the network it was connected to remain active for the maximum possible duration, allowing a study to focus on one culture and yet continue to get the amount of data needed for clear results.

This solution was simple and versatile enough to be scaled to fit any number of neuron sizes, from the 50-100µm diameter neurons of a snails nervous system used by this study to the 20-30µm diameter neurons of a rat’s hippocampus used in others. The simplicity of the clusters allowed for these scales to be quite within the current’s technology’s reach. Though a great help to researchers, this apparatus did have its drawbacks. A neuron network is a system that is always physically rearranging itself as it reconfigures its synapse connections. This is one of the reasons why such a hybrid system is desirable – it is a network that adapts as it learns. Of course, this phenomenon is still under study, and hopefully these very same tests can help to shed some light on how these physical orientations relate to the signal and how it is transferred along the network. Of course, the silicon substrate does not have this same property, which means that as the cells grow in the culture they tend to rearrange themselves with no regard to the locations of the stimulator-sensor clusters. This leaves the researchers with batches partially or entirely unusable, with fully formed networks that they are unable to test without dooming it to death within a few hours.
While this completely planar method allowed for some very useful results, its drawbacks made the tests expensive and time consuming. As with any preliminary design, each subsequent iteration made an effort to solve the problems of its predecessor. The primary concern at this point was the mobility of each carefully placed neuron, which could reduce the yield from 6-10 testable cells to a gamble that can yield none at all.

The first reiteration of this design (Zeck G, Fromherz P, 2001) deals with this problem very simply. By building polyimide picket fences around each stimulator-sensor cluster each neuron is confined in its mobility though its processes are able to grow out in every direction, free to connect to other neurons and create a testable network that remains through the life of each neuron.

The manufacture process is hardly more complicated than the planar interface. Aside from the slight difference in the array’s structure, the only addition to the original design in the inclusion of 5 polyimide posts to act as a fence around each cluster. The considerations of the design included array orientation, post spacing, and post height. The pull of the neurons when they are connected in a network can be enough to pull the body of the cell between two posts, even at the expense of the cell’s life. While this can be a problem, for this setup that happens very rarely and even then it is a vast improvement over the yield of the past design.

The signals that are recorded in this set up are identical in quality and superior in frequency to the planar design. These improvements have allowed valuable data to be collected and have contributed greatly to the goal of complex neural-microelectronic systems in the future. Of course these designs can and have been improved upon in various uses, where the neuron would have higher frequency of pulling itself through the posts, such as the case of rat’s hippocampal neurons which are both smaller in size and more forceful in their movement. These problems have been addressed in other designs that have been developed in other laboratories along a similar timeline.
Part II: Small Neuron Network by Cages on Chip [3-5]

Introduction

Studying how neural networks, formed by individual neurons, perform in detail is the central task of neuroscience. The conventional technique is to use planar arrays of extracellular metal electrodes on which neural cultures are grown. However, this approach can only arbitrarily select and access a small proportion of neurons in the entire network. Furthermore, since neurons are mobile, repeated measurements of a specific neuron are difficult to obtain, especially for long term experiments.

In order to study each neuron in the neural network, one-to-one correspondence between the neurons and electrodes has to be established and maintained. This can be achieved by physically confining individual neurons over corresponding electrodes without affecting neurite growth and neural network formation. To accomplish these goals, micro-cages can be used, trapping each neuron into one cage while still allowing neuritis to grow out. The neuro-cages are constructed in arrays to allow neurites from different neurons to form neural networks. Thus the cultured neurons in a network can be reliably stimulated and monitored individually and continuously over long periods of time.

Silicon micromachined neurochips

The neurochip is a 1 cm square, 500 μm thick silicon wafer (thinned to 16 μm over a 4x8 mm² area in the center) with a 4x4 array of wells spaced on 100 μm centers. Fig. 1 illustrates the structure of the neurochip at three different length scales. The building block of the neurochip is the neurowell, which is designed to trap the cell body of a single neuron next to the electrode. The depth of the neuron well, or the thickness of the membrane (20 μm), is chosen to accommodate the size of the neuron target when fully grown. Fig. 2 shows an SEM photograph of a fabricated neuron well from the cavity side. Clearly seen is a heavily boron-doped silicon grillwork on top of the well and a circular gold electrode at the bottom.
Fig. 1: Design and dimension of a neurochip.

Fig. 2: SEM of a single neuron well.
Fig. 3 shows how the neuron implanted in the well can grow out its neurites.

Microfabrication Process

Fig. 4 schematically represents the fabrication process. The fabrication starts with epi-wafers with a 16 µm lightly-doped layer on top of a 4 µm heavily boron-doped layer. First, a composite layer of 180 nm LPCVD silicon nitride on top of 50 nm thermal oxide is formed. Photolithographic steps then pattern the nitride-oxide layer to define the openings (6 µm in diameter) for the metal electrodes at the bottom of the neuron wells. Plasma and isotropic silicon etching steps then follow to etch through the nitride –oxide composite layer together with a 0.5 µm recess into the silicon substrate. Then 1 µm oxide step is created around the electrode openings. The purpose of this is to ensure good electrical isolation of the electrode and to provide a tight seal to the cultured neuron in the well. The nitride is then stripped and 200 nm Au metallization is done using a lift-off process. This metallization is covered by a composite insulation layer of 0.5 µm LTO and 1 µm PECVD nitride. This combination is used for stress compensation. Next, opening of bonding pads and etching of alignment marks in the insulation layer are performed. The alignment marks are used later to do front-to-back alignment across a membrane that is micromachined in the subsequent steps. In fact this technique allows alignment within 1 µm, which is crucial for placing the electrode opening right at the center of each neuron well. Then windows on the back of the wafer are opened. The following EDP etching then forms the silicon membrane using the heavily boron-doped buried layer as an etch stop. Photolithographic steps on the cavity side of the membrane follow, and RIE etching is used to form grillwork. The neuron wells are formed by EDP etching all the way to the
well electrodes on the front side of the wafer. The removal of pad oxide at the bottom of the wells then finishes the fabrication.

Fig. 4: Microfabrication process.

Experimental results

A process has been developed to implant embryonic neurons into the wells without damaging them using micropipettes. This procedure is shown in Fig. 5. Major steps in the process of moving embryonic neurons into the wells are: a) Neuron sucked and held in pipette while being moved to a well b) Cell ejected from the pipette near a well c) Pusher positioned to move cell over the well d) Cell implanted in the well by a pusher. The process shows a survival rate of 75%.
As much as three quarters of the neurons pushed into the wells can grow neurites out of the wells. Moreover, the biocompatibility of the neurochip is evident for these implanted neurons further grow into live neural networks, as Fig. 6 shows.

Parylene neurocages

Open-faced neuro-wells have shown several drawbacks. First the process of making bulk micromachined wells is very complicated. In addition, neurites growing out through the
top of the well tended to pull neuron away from well-bottom electrodes. To address these issues, a more suitable technology using Parylene and thick photoresist was developed incorporating a new structural design consisting of long thin channels radiating from the base of the cage. The structure of the new surface-micromachined neuro-cages is illustrated in Fig. 7.

![Fig. 7: 3D illustration of the neurocage.](image)

Parylene is chosen as the structural material in this application for its unique properties. It is non-toxic, extremely inert, resistant to moisture and most chemicals, and biocompatible. These properties make parylene well suited for long-term cell culture experiments. Its conformal deposition makes it easy to fabricate 3D structures like the neuro-cage, thus simplifying the fabrication process as compared to the bulk micromachined neuro-well. Most importantly, parylene is transparent. Thus neurons can be seen under microscope through the parylene cages and neurites are easily observed as they grow through the channels. The cage consists of a top loading access hole, the cage body, and 6 thin channels that protrude from the bottom of the cage, as Fig. 8 shows.

![Fig. 8: SEM picture of a fabricated neuro-cage.](image)
Microfabrication process

The generalized fabrication flow is shown in Fig. 9. First an oxide layer is grown on silicon wafer. A channel height controlling sacrificial layer is the patterned. Two parylene layers and one photoresist layer are used to form the cage. The sacrificial materials are finally removed to release the microcage.

Efforts have also been made to improve the adhesion of the neuro-cages to the substrate. While parylene-to-oxide adhesion is usually improved by applying A-174 to the substrate before parylene deposition, this is insufficient for withstanding immersion in aggressive chemicals used in sterilization and cell culture solutions for long periods of time. Therefore two alternative robust adhesion promotion techniques are investigated. The first method relies on mechanically anchoring parylene to the silicon substrate. The other technique is to roughen the anchoring area with short time etching in BrF3 or XeF2.

The filled-trench process (Fig. 10) starts with patterning oxide layer on silicon wafer to expose silicon in the trench/anchor areas. Then 0.3 µm silicon and 0.2 µm Al are sputtered. First, Al is patterned and chemically etched. Then sputtered silicon is patterned and etched in DRIE using photoresist and Al as mask. DRIE is then used to make the anchoring structures. The standard procedure is used to etch a 10 µm deep trench. Then an isotropic etching in SF6 plasma is performed to create a widened portion at the bottom of the trench. Parylene is deposited and patterned followed by sacrificial materials removal. Fig. 11 shows the fabricated neuro-cage.
The roughening process also starts with patterning oxide to expose silicon in the anchor areas. A brief etching in XeF$_2$ gas is then used to roughen the silicon surface. The illustration and fabricated cage is shown in Fig. 12.
Experimental results

First, parylene has been shown to be compatible with neurons. Fig 13 shows the growth of neurons on a parylene surface. There is no observable difference between neural growth on parylene and oxide surfaces. Neurons are observed to grow multiple processes that are long and branching, which is an indication of healthy cells. Also, neural networks were formed.
Secondly, the parylene neuro-cages are shown to be functional as designed. Freshly dissociated neuron can be loaded into neuro-cage. Neurites have successfully grown out from cage channels, while the neuron cell body is still trapped inside the cage.
Fig. 13: Neural growth on parylene surface.
Part III: Building Living Neuron Chip with Peptide Amphiphiles Gels [6-9]

After considering about previous two types of neuron chips for monitoring neuronal electrical circuitry, we propose a new type of neuron chip for the same purpose here, which will be easier to fabricate and can be used to make larger neuron network. It is a combination of MEMS technique with nanotechnology. First we can use soft lithography to make a pattern on a Poly(dimethylsiloxane) (PDMS) substrate according to our design. Neuron cells trapped within peptide amphiphiles gels with neuronal specific epitope IKVAV peptide sequence will be selectively delivered to this micro fabricated wells array. With the help of this IKVAV PA gels, cells can be fixed within the gels. If we have some opening between wells, cells can have contact there. This design combines physical and chemical factors together for patterning neuron cells. Once succeed, it can provide a very simple way to build a large scale neuron network. Fig 14 below gives the illustration of this idea.

![PA gels with trapped cells](image_url)

Fig 14 Novel Neuron Chip with PA gels
Several issues to consider about

Peptide Amphiphiles

Peptide Amphiphiles (PAs) shown in Fig 15, synthesized in the Stupp laboratory by postdoctoral researcher Jeffery Hartgerink, have been found to self assemble into gel forming cylindrical micelles. The molecular building blocks of these nanostructures consist of several distinct regions that endow these molecules with their self assembling nature. On one end, a long alkyl tail gives the molecule a hydrophobic nature. Connected to this tail is a series of four peptides that can be used to covalently lock in the micellar nanostructure through disulfide bonds between adjacent cysteine residues. Alternatively alanine residues may be used here if no crosslinking is required. Next to this region is another series of peptides designed to provide flexibility between the rigid covalently linked region and the hydrophilic peptide cap containing the biologically significant peptide sequence. These molecules are capable of incorporating a number of biologically relevant peptides that can directly interact with cells, such as the common adhesion peptide RGD, or the neuronal adhesion peptide IKVAV.


Fig 15 Peptide Amphiphiles
**Replica Molding of PDMS**

Poly(dimethylsiloxane) (PDMS) is an appropriate substrate for such purpose. Because it is optically transparent down to 300 nm, has been shown to support cellular attachment and growth, and opens up routes to microwell fabrication. PDMS is a silicone elastomer commercially available in 1.1 lb polymer/curing-agent kits from Dow Corning under the trade name Sylgard Elastomer 184, each kit selling for approximately $40. PDMS may be readily used in a technique known as *replica molding* to rapidly create high aspect ratio topographical features in the elastomer with microscale resolution. Fig 16 shows the process for making such molds.

![Replica molding](image)

**Fig 16 Process for making PDMS molds**

**Selective delivery by Membrane Patterning**

The way for selective delivery of cells trapped inside of PA gels can be described in Fig 17 below. A membrane of the same pattern as the substrate is fabricated by spin coating of the PDMS precursor followed by baking at 100 °C for 45 minutes. The membrane mask is then aligned to the substrate with a registration station. Cells and PA gels are
introduced into the wells through the membrane mask. After removing the membrane mask, the whole system can be submerged under culture media for culturing.

In order to make the process easier, we can make the mask and substrate on the same sample, by multilayer spin coating, as illustrated by fig below. After the demolding of the device the membrane will be self registered to the substrate. (Note, there will be some separation layer between the silicon wafer and the PDMS layer and also between the membrane layer and the bulk materials.)

Fig 17 Self Registed Membrane Patterning Technique
Reference

2. Martin Jenkner, Bernt Müller, Peter Fromherz , Interfacing a silicon chip to pairs of snail neurons connected by electrical synapses, Biological Cybernetics 84, 239-249 (2001)
4. Qing He., Ellis Meng, Yu-Chong Tai, Christopher M. Rutherglen, Jon Erickson, and Jerome Pine, Parylene Neuro-Cages for Live Neural Networks Study, The 12th International Conference on Solid-State Sensors, Actuators and Microsystems, (Transducers'03), Boston, MA, Jun 8-12, 2003