MEMS based examination platform for neuro-muscular communication in a co-culture system coupled to a multi-electrode-array

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ABSTRACT

Development of sensor-connected in vitro neuromuscular co-culture systems may provide a useful tool for interpreting mutual signal transfers between the cell species and for future drug screening applications. A glass-based, autoclavable, micro fluidic culturing system consisting of two chambers connected by 50 µm micro capillaries with a diameter of 5 µm was designed. Micro capillaries were generated by two technological approaches. First reactive ion etching was used for structuring the glass capillaries as well as a subsequent bonding to a glass carrier with electrodes by using an UV-activatable adhesive. As second approach a structured SU8-layer was used simultaneously for generating the capillaries and as an intermediate layer for bonding. Chambers and fluidic luer connectors (structures in sub-millimeter scale) were realized by means of a cost effective micro-sandblasting process. The co-culture system was combined with a commercially available multi electrode array. This setup allows stimulation of adherent neuronal cells in one chamber and measurement of action potentials induced in myotubes derived from the myogenic C2C12 cell line in the other. Neuronal processes growing through micro capillaries were detected using immuno-fluorescence staining methods. This hybrid technology represents a new approach for electrophysiological recordings.

Keywords: bio-mems, neuromuscular synapse, mea, co-culture

1 INTRODUCTION

Many aspects about the factors [1] that facilitate the interplay between motoneurons and the muscle fibers innervated have been unravelled by examining tissue specimen or studying different types of co-culture models [2- 4]. Our co-culture model intends to generate motorical end plates derived from cell lines. To examine how these are developed and what type of substances may activate or inhibit this process, electrophysiological recordings of neuromuscular interactions are surveyed on multielectrode arrays. This technique allows to measure neuromuscular activities in spatiotemporal resolution. In the system presented here (Figure1), a commercially available planar multielectrode array (Multi Channel Systems MCS GmbH, Reutlingen, Germany) with 64 electrodes has been combined with a co-culture system that facilitates separated growth of monolayers of neuronal and myogeneic species. Additionally, stimulation of the adherent cells and subsequent recording of the extracellular signals will be examined.

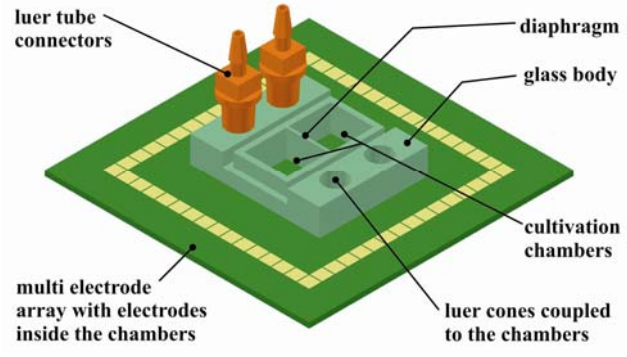

Figure 1: Scheme of the co-culture system

2 TECHNOLOGICAL SETUP

As to obtain a chamber volume of about 300 µl at lateral geometries defined by the MEA, a minimum chamber height of 5 mm is necessary. Glass with an adequate UV-transmission was chosen as construction material to achieve a comfortable illumination for fluorescence microscopy. Therefore a 100 mm wafer-formatted borofloat glass substrate with a thickness of 5 mm was used allowing standard MEMS technologies. Two technological approaches, Reactive Ion Etching (RIE) and SU8-lithography were tested to manufacture the lateral micro capillaries on the bottom of the diaphragm.

2.1 Fabrication of the glass body

To create 5 mm deep structures in glass substrates, micro sand blasting is an effective method with an adequate lateral accuracy and a high ablation ratio compared to plasma etching techniques. Thin steel masks, patterned by lithography and etching are aligned and fixed on both sides of the glass wafer using a bond wax. A compressed air beam containing 30-µm silicon carbide powder is used to scan both sides of the masked glass surface until the

chamber structure is broken through. Figure 2 depicts the glass wafer after removing of the steel masks. A grinder fitting the angle of the luer cones was utilized to refinish the connecting holes.

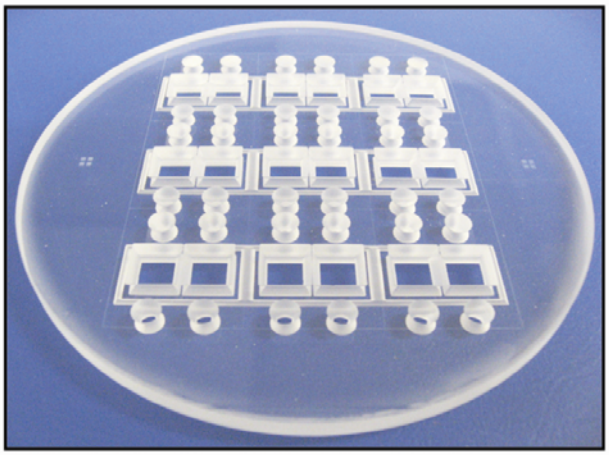

Figure 2: Micro sand blasted glass wafer

2.2 Fabrication of the capillaries using RIE and gluing

As a first approach the micro capillaries which have a dimension of 5 µm x 5 µm x 50 µm were generated by a modified RIE process before micro sand blasting of the glass wafer. A parallel plate reactor (STS 320) with a cooled wafer electrode and a CHF_3 / CF_4 – plasma was used. Thereby an etched chromium layer patterned by a lithography step acts as masking layer. Figure 3 depicts the bottom side of the glass wafer where the micro capillaries are patterned. After the etching process the chromium layer was removed.

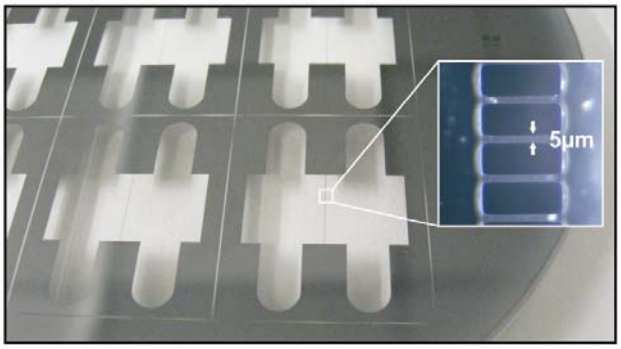

Figure 3: 5-mm glass wafer structured with RIE

Hereafter the processed glass wafer was sand blasted as mentioned above. So as to separate the single glass bodies of the glass substrate standard wafer dicing was used. The bonding procedure of the glass body to the MEA-system was accomplished by gluing. With a microscope the MEA-system and the glass body are aligned and fixed by a clamping mechanism. Using capillary forces the small gap between the two glass components was filled with a liquid UV-cured acrylat adhesive (SurABond HH 051), stable at higher humidity and temperature. Because of the excellent UV-transmission of both glass components the exposure was very simple.

However, after various evaluations, particular after few autoclaving tests and several changes of the medium the glass bodies were de-bonded. Because the overall system is designed for multiple uses it was necessary to verify the second technological approach.

2.3 Fabrication of the capillaries using SU8-lithography and bonding

For simultaneous creation of the 5-µm capillaries as well as the intermediate bond layer SU-8 [5], an epoxy based photoresist was used. This resist from MICRO-CHEM is commonly used for micro electro mechanical systems (MEMS) with high aspect ratios as well as high chemical and thermal stability. The lithography step was directly implemented on the MEA glass carrier. The 49 mm x 49 mm glass substrate with electrodes and a passivation layer was coated with 5-µm thick SU8-2005 layer and patterned by a modified lithography step (Fig. 4a). After an adapted cleaning step, including plasma treatment a single co-culture glass body was aligned and pressed to the MEA glass carrier. Using a heating press with an adequate parallelism a very stable bonding between glass body and MEA system was achieved during the post exposure bake of the resist at 110 °C.

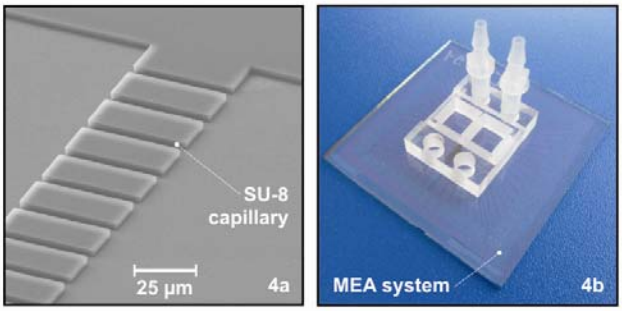

Figure 4: Patterned SU-8 layer before bonding (a), completely bonded co-culture system (b)

3 CELL BASED APPLICATIONS

3.1 Cell Culture

Undifferentiated NG108-15 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St.Louis, MO, USA) supplemented with 10% FCS + Penicillin and Streptomycin + L- Glutamine. To induce the differentiation, the culturing medium was changed to serum free DMEM + Penicillin and Streptomycin + diButyryl- cyclic AMP (dBcAMP, Sigma-Aldrich). C2C12 myogeneic cells (ATCC) were maintained in DMEM + 10% FCS + Penicillin and Streptomycin + L- Glutamine.

Upon changing the culturing medium to DMEM + 2% heat-inactivated horse serum (PAA, Austria), differentiation to myotubes was observed from 5 DIV. MEA dishes were coated with 0.02 % gelatine.
2.5 x 10^4 undifferentiated C2C12 cells were plated to one chamber of the co-culture system and cultured for 9 days. Undifferentiated NG108-15 cells were added to the other chamber. Both cell species were maintained in DMEM + 2% horse serum.

3.2 Transfection

NG108-15 cells were transiently transfected either with the pDsRed-Express-C1 (Clontech, Mountain View, CA, USA) or pZsGreen1-C1 vector (Clontech, Mountain View, CA, USA), respectively. For this process, Lipofectamine 2000 Kit (Invitrogen, Karlsruhe, Germany) was used according to the protocol provided by the supplier. All the expression plasmids contained a G418- resistant gene as described by the supplier. G418 stable cell lines were selected in medium containing 400µg/ml G418 (Geneticin, Invitrogen, Karlsruhe, Germany) and tested by expression of pDsRed or pZsGreen1. Successfully transfected NG108-15 cells were sorted using a Partec CyFlow®space flow cytometer connected to a Partec Particle and Cell Sorter (Partec GmbH, Muenster, Germany).

3.3 Immunohistochemistry

Neuronal processes were stained with antibodies directed against microtubule- associated protein 2a (FITC labelled MAP2a, clone AP20, Abcam, Cambridge, UK), growth- associated protein 43 (GAP43, clone GAP-7B10, Abcam, Cambridge, UK) or neurofilament 200 (NF-H, clone E15, Santa Cruz, CA, USA), respectively. Cells were washed and fixed with ethanol for 5 minutes. Nonspecific binding was blocked using 10 % normal goat serum (PAA, Austria) in PBS. Cells were incubated with primary antibodies at 4°C overnight. After rinsing, cells were exposed to Texas Red labelled donkey anti goat IgG or goat anti-mouse IgG at 4°C, dark and overnight. Cells were examined with an inverted Nikon Eclipse TS100 microscope (Nikon, Japan) equipped with appropriate filters and camera (VDS Vosskühler, CCD1300CB, VDS Vosskühler, Germany). Fluorescent photomicrographs were taken with a magnitude of 40. The images were then processed (Adobe Photoshop, Mountain View, CA, USA) as appropriate for presentation in the figures.

4 RESULTS AND DISCUSION

A commercially available planar multielectrode array (Multi Channel Systems MCS GmbH, Reutlingen, Germany) with 64 TiN electrodes was combined to a glass co-culture system consisting of two cultivation chambers interconnected by micro channels of 5µm width and 50 µm length. These channels serve as a diaphragm. This arrangement enables neuronal processes to grow through the channels. The area size of one chamber comprises 65 mm^2. As the system is autoclavable, it is suitable for repeated measurement approaches. The electrodes are directly located beside the diaphragm (left and right). Via Luer fittings, the co-culture system may be connected to a perfusion system, thus allowing both static and fluidic cultivation conditions. We established a co-culture of the neuroblastoma x glioma cell line NG108-15 [6] and mouse myoblast cell line C2C12 cells [7]. NG108-15 cells have been reported to resemble motoneurons as they are able to synthesize acetylcholine, agrin and neuregulin [8, 9]. These cells are capable of generating functional synapses with myotubes including induction of AChR clusters [8, 10]. Moreover, twitching of myotubes cocultured with NG108-15 neuronal cell has also been described [8]. C2C12 cells provide a well established system to cultivate myotubes and produce acetylcholine receptors. Dye conjugated alpha-bungarotoxin, a snake venom with high binding affinity to nicotinic acetylcholine receptors, was used to visualize attachment of neuronal cells to myotubes (Fig. 5).

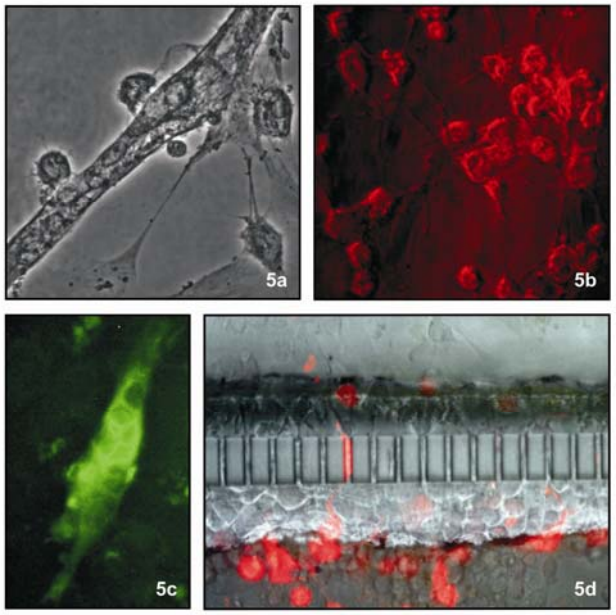

Figure 5: Neurites of NG108-15 cell binding to C2C12 myotubes (a). These cells express neurofilament –H (b) and MAP2a (not shown). Positive staining of FITC conjugated α-bungarotoxin demonstrates the presence of nACh receptors (c) in C2C12 myotubes. Neuronal processes of pDsRed transfected NG108-15 cells growing through microchannels separating the cultivation chambers (d).

Some neurological disorders are correlated with a defective signal transmission between the innervating motoneuron and muscle fibers. Therefore, we are seeking to further understand the electrophysiology of the neuromuscular interface. Our MEA- based co-culture system may provide

a new setup for electrophysiological recordings of the cell culture derived end plate potentials.

5 ACKNOWLEDGEMENTS

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