Integration of living cells with silicon nanostructures for MEMS applications

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ABSTRACT

For many years biological and silicon-based structures were considered incompatible because of the difficulties to engineer an effective interface connecting the two systems. Recent developments in surface chemistry, bioengineering and cell biology enabled the creation of extracellular clues to guide the attachment and growth of cells on silicon structures. We have developed a photolithography-based method, which was compatible with standard silicon manufacturing steps, to pattern self-assembled monolayers (SAMs) on glass or silicon substrates to guide cell attachment, pattern formation and differentiation. In order to demonstrate the effectiveness of our method to create functional cellular networks on silicon microstructures we have registered the surface patterns with substrate embedded micro electrodes to allow long-term recording of activity of the cells or electrical stimulation. These hybrid biological/silicon-based systems can find applications in functional pharmacological screening, toxin detection, as disease models or in robotics.

Keywords: cell patterning, engineered neuronal networks, skeletal muscle engineering, self-assembled monolayer, photolithography, MEMS, cell-electrode interface, multi-electrode arrays

1 INTRODUCTION

One of the most promising application areas of nanoscience and nanotechnology is biomedical research and industry. Manipulating and organizing living cells is a central problem in several interdisciplinary scientific areas such as tissue engineering, biotechnology, biosensors or Micro Electro Mechanical Systems (MEMS) technology. In contrast to conventional engineering and manufacturing, living systems can not be manipulated directly using conventional methods. Instead, new technologies should be developed which are using the internal programs of the cells and their innate self-organizing capacity to create complex functional cellular systems [1, 2]. This new technology is facing two major challenges. One of them technical: the development of the technology for the presentation of extracellular (soluble and surface bound) signals to the cells in a time and spatially dependent manner [3, 4]. The other challenge is related to our limited knowledge of the state and the internal programs of the cells.

The interest of our laboratory is the creation of simple functional cellular assemblies from dissociated cells which mimic specific properties of the intact organism. The major application areas of these engineered biological systems are functional pharmacological screening, toxin detection, *in vitro* disease models or robotics.

We have developed a photolithography-based method to pattern self-assembled monolayers (SAMs) on glass or silicon substrate to guide cell attachment and differentiation [5-7]. This method is fully compatible with modern silicon manufacturing steps. Surface modification with self-assembled monolayers is an exceptionally flexible technique to determine the characteristics of surfaces [8, 9]. Moreover, SAMS can be used to immobilize proteins in a spatially controlled manner without denaturation [10]. We have demonstrated earlier that several cell types can be cultured in serum-free medium on SAMs in a defined system [11-14].

The goal of this study was the development of the methods necessary to integrate living cells with silicon structures for MEMS applications.

2 METHODS

2.1 Surface modification with SAMs

Glass coverslips were cleaned using HCl/methanol (1:1), soaked in concentrated H_2SO_4 for 30 min then rinsed in dd.H_2O. Coverslips were boiled in deionized water, rinsed with acetone then oven dried. The trimethoxysilylpropyldiethylenetriamine (DETA, United Chemical Technologies) film was formed by reaction of cleaned surfaces with 0.1% (v/v) mixture of the organosilane in toluene. The DETA coverslips were heated just below the boiling point of toluene, and then rinsed with toluene, reheated just below the boiling temperature, and then oven dried. Surfaces were characterized by contact angle and X-ray photoelectron spectroscopy methods [13]. In some experiments (skeletal muscle patterning) DETA coated coverslips were incubated 10 µg / ml (in water) vitronectin (Invitrogen)

2.2 Photolithography

Quartz photomasks were designed using the CleWin layout editor (WieWeb, Hengelo, The Netherlands) and fabricated through a commercial vendor (Bandwidth Foundry Pty Ltd., Australia). Surface of the coverslips not

covered by the photomask were ablated using a 193 nm Ar/F LPX200i laser beam (Lambda Physik, Ft. Lauderdale, FL) combined with a beam homogenizer (Microlas, Ft. Lauderdale, FL, Energy density: 50 mJ/cm^2) to create the surface patterns.

2.3 Cell culture

Embryonic hippocampal cultures

Hippocampal cells were obtained from day 17 rat embryos. The hippocampi are collected in cold Hibernate E+ glutamine 0.5mM+ B27 supplement. The tissue was triturated using a sterile pasteur pipette. The dissociated tissue was centrifuged at 1000 rpm for 2 minutes. The pellet was resuspended in growth medium. The growth medium consisted of Neurobasal (Invitrogen 21103-049) glutamine 0.5mM+ B27 supplement. The cells were plated on the pattern of the SAMs at a density of approximately 60- 120 cells/ mm^2 in the serum-free culture medium.

C2C12 culture

C2C12 cell line was obtained from ATCC. Cell stock was grown in T-75 flasks in DMEM (HyClone, SH30243.01) + 10% Fetal Bovine Serum at 5% CO_2 and 37°C. After confluence, the cells were dissociated in Cell Dissociation Solution (HyClone, HyQTase) by tituration in a 5 ml pipette. After centrifugation (500g, 5 min) C2C12 cells were replated in differentiation medium (DMEM+ 1% B27 supplemet, Invitrogen) on the glass coverslips.

Embryonic cardiac culture

Cardiomyocytes were obtained from rat embryos on the 14th day of the embryonic development (E14) [15] Briefly, rats were euthanized by inhalation of an excess of CO_2. The hearts were removed from the embryos in Hibernate E medium and dissociated using type II Collagenase (Worthington, LS004174, 125 units/g, 1 g/5 ml) in L-15 medium. The hearts in the collagenase solution was placed in the water bath (37 °C, 90 rpm) for 20 minutes followed by gentle manual trituration. The cell suspension was then centrifuged on a 4 % Bovine Serum Albumin (BSA, Sigma, A-3058) cushion at 300 g, 4°C for 10 minutes. The cell pellet was then resuspended in the culture medium and plated on the surface-modified coverslips at a density of 1000 cells/coverslip. The culture medium consisted of 100 ml of Ultraculture medium (Cambrex), 5 ml of B-27 (Invitrogen), 1 ml of non-essential Amino Acids (Invitrogen, 11140-050), 1 ml of L-Glutamine (Invitrogen, 25030-164), 1 ml of 1 M Hepes Buffer (Sigma), and 0.375 g of dextrose (Sigma). The medium was changed on the first day after culture and thereafter every 3 days.

All cell culture experiments were repeated 3 times with 3 coverslips in each experiments.

2.4 Electrophysiology

The microelectrode arrays and accompanying accessories, including the temperature controller, stimulator, amplifier and MC_Rack V 2.2.2 data acquisition software were obtained from ALA Scientific (Westbury, New York) and Multichannel Systems (Reutlingen, Germany). The microelectrode arrays were comprised of a glass base that acted as a substrate, gold connector contacts and electrodes composed of titanium nitride. Pyrex glass rings were affixed to the microelectrode array using Sylgard 184 (1 part curing base and 10 parts elastomer base) to form the culture chamber around the microelectrodes. The hippocampal cells on the microelectrodes were bathed in culture medium and maintained at 37°C for electrophysiology readings. Parallel multi-site electrophysiology and network analysis was done using the MEA-60 system. Online sampling and data analysis was done both real-time and with stored data.

3 RESULTS

We have successfully demonstrated that different cell types can be patterned using the same technology: photolithographic patterning of self-assembled monolayers. We have optimized the pattern design for single-cell patterning of embryonic hippocampal cells (Figure 1). The optimal pattern size was 200 µm with 2 µm line width. Using patch clamp electrophysiology we have demonstrated that patterned hippocampal cells showed normal physiology and formed functional synapses on the patterns.

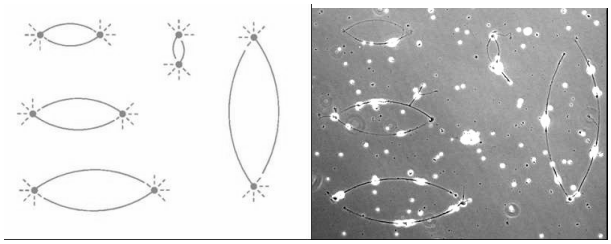

Figure 1. Optimization of two-cell neuronal networks. A combination pattern was created with different feature sizes and line width. Left: photomask design, Right: phase-contrast picture of embryonic hippocampal cells growing on the patterns. The distance between the cell attachment sites on the largest pattern is 500 µm.

We have also demonstrated that surface patterns can be registered with substrate embedded electrodes. Hippocampal cells plated on the patterns formed directed synaptic connections. Multielectrode extracellular electrode recordings showed that functional neuronal networks formed (Figure 2).

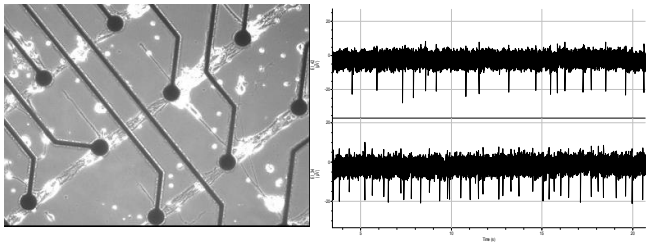

Figure 2. Registration of neuronal networks with substrate-embedded microelectrode arrays. Left: phase-contrast picture of embryonic hippocampal cells are growing on the top of the electrodes and making connections between the lines. Right: extracellular electrophysiological recordings from patterned neurons. Electrode spacing: 200 μm.

For the patterning of skeletal muscle (C2C12 skeletal muscle cell line) a different strategy had to be developed, because in our serum-free defined culture system differentiation of C2C12 cells into myotubes required surface-bound signals such as substrate adsorbed vitronectin or laminin. Based on this substrate-requirement of myotube formation, we developed a photolithography-based method to pattern C2C12 myotubes, where myotubes formed exclusively on vitronectin surface patterns. We have determined that the optimal line width to form single myotubes is approximately 30 μm. In order to illustrate a possible application of this method, we patterned myotubes on the top of commercial substrate-embedded microelectrodes (Figure 3).

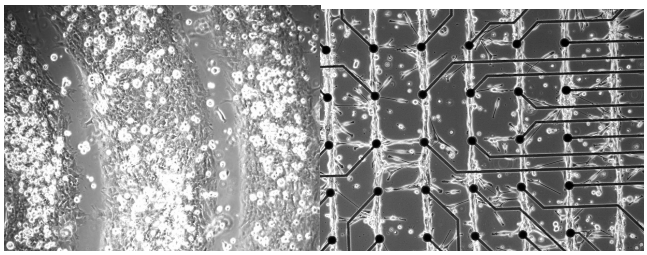

Figure 3. Photolithographic patterning of cardiac myocytes (left) and skeletal muscle myotubes (right). Phase contrast pictures. The cardiac myocytes were plated on 150 μm wide lines. Electrode spacing is 200 μm.

Patterning of cardiac myocytes was the most challenging problem, because primary cardiac cells poorly attached to the DETA surface. We have determined that with this cell type the inverse-strategy was the most effective, namely embryonic cardiac myocytes formed patterns on the ablated surface (Figure 3).

4 DISCUSSION

We have shown that a vide variety of cell types can be patterned using photolithography and self-assembled monolayers on silicone / glass substrates. These patterns can be registered with micro- and nanostructures on the surface. Our method is compatible with standard silicon manufacturing steps and an effective tool to integrate cells into hybrid devices. In our laboratory this method is being further developed for applications in cell biology, tissue engineering and robotics.

5 ACKNOWLEDGEMENT

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