# Reflex-arc on a chip: An *in silico* cell culture analogue

K.A. Wilson, M. Das. P. Molnar, J.J. Hickman

NanoScience Technology Center, University of Central Florida, 12424 Research Parkway Suite 400, Orlando, FL, USA,
kwilson@mail.ucf.edu, mdas@mail.ucf.edu, pmolnar@mail.ucf.edu, jhickman@mail.ucf.edu

## ABSTRACT

Treatment and understanding of traumatic spinal cord injury (SCI) and neurodegenerative diseases have been problematic due in part to difficulties associated with the various models used to test new drug therapies. Animal studies are expensive, time consuming, and can raise ethical issues. *In vitro* studies avoid many of the issues associated with animal studies and can allow the use of human cells, but such data are too incomplete to be predictors of human response due to the lack of interaction between tissues and differences between culture and physiological dynamics. To overcome the shortcomings of existing models we are developing a microscopic cell culture analogue (microCCA) of the spinal reflex-arc. This system will provide a model which can accurately reflect important aspects of *in vivo* behavior of animal models, but retain the cost effectiveness of *in vitro* models as well as the ability to perform large numbers of tests in parallel

***Keywords***: MEMS, silane, muscle, cell culture, neuron.

## 1 INTRODUCTION

The present work draws on advances in a wide variety of technical fields including cell culture, surface chemistry, and microfabrication. These advances have enabled the development of microCCA devices for high throughput studies of cell/cell, cell/tissue, tissue/tissue interactions with particular emphasis on drug discovery [1]. We are constructing a microCCA device comprised of the basic components of the reflex-arc: a muscle fiber, a dorsal root ganglion (DRG) cell, and a motoneuron. These components will then be cultured on a microfabricated silicon cantilevers. Using surface modification techniques developed by Ravenscroft and coworkers [2] we have demonstrated the ability to direct cell growth and create cellular networks on silica substrates. Also, we have developed a defined serum-free media (L15 media) that has allowed the co-culture of neurons and muscle cells. In this way we are able to culture cell types from opposite sides of the blood-brain barrier. With this system it will ultimately be possible to report on a variety of parameters of the reflex-arc, such as cellular membrane potentials, synapse formation and contractile forces produced by the muscle fiber, thereby creating a cost-effective, predictive test bed for the study of traumatic SCI and a wide variety of neurodegenerative diseases.

## 2 MATERIALS AND METHODS

### 2.1 Surface Modification

Trimethoxysilylpropyl-Diethelynetriame (DETA) from United Chemical Technologies (Boston, MA) was diluted in dry toluene to a 0.1% (v/v) working solution. Glass coverslips and silicon cantilevers cleaned in $O_2$ plasma were then immersed in the DETA solution and heated to approximately $60^0$C for 30 minutes. The reaction vessel was allowed to cool for 30 minutes. After cooling the coverslips and cantilevers were washed 3x in dry toluene, followed by a second heating step in dry toluene for 30 minutes. After the second heating, coverslips and cantilevers were baked overnight at $110^0$C.

Photolithography was performed on DETA coated coverslips according to Ravenscroft et al [2]. Briefly, the coverslips were placed on a mask aligner and covered with a quartz/chromium photomask. The coverslips were then irradiated with 193 nm deep UV radiation for 30 seconds (250 mJ pulses at 10 Hz) to ablate away DETA in the exposed regions of the coverslip.

Ablated coverslips were then backfilled with (Tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane (13F), purchased from Gelest (Morrisville, PA), to form the cytophobic background surface. A 0.1% v/v dilution of 13F was made in dry chloroform in which coverslips were immersed for 5 minutes. Coverslips were then washed 3x in fresh dry chloroform then baked overnight at $110^0$C. Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Cam 200) and by X-ray photoelectron spectroscopy (Kratos Axis 165) by monitoring the N 1s and F 1s peaks.

### 2.2 Cantilever Fabrication

The fabrication process for the devices in Figure 1 was straight-forward. The design for the cantilevers was generated using AutoCAD 2004, and was used to create the photomask which consisted of a 5 inch square quartz plate coated with chromium. The cantilevers were fabricated from Silicon on Insulator (SOI) wafers using a deep reactive ion etching (DRIE) process. A double-sided polished 10 µm thick crystalline silicon wafer was bonded to a 500 µm $SiO_2$ handling wafer and the crystalline silicon surface was coated with a 1.3 µm layer of AZ 5214

photoresist. The photoresist was exposed to a soft bake followed by contact exposure with the mask. The photoresist was then developed, hard baked, and the wafer was mounted on a 6" handling substrate for DRIE. After DRIE, the photoresist was removed via a wet strip followed by plasma cleaning. The wafer was cut into 10 mm x 10 mm pieces, containing the cantilever arrays, by dicing followed by HF release and supercritical $CO_2$ drying. The processing was contracted through the MEMS Exchange of Reston, VA. A rectangular cantilever was used so that the spring constants could be easily calculated and it was hoped that the shape would aid and direct in the adhesion of the myotubes.

## 2.3 Isolation and Culture of Reflex-arc elements

Muscle Preparation

The skeletal muscle was dissected from the thighs of the hind limbs of E18 rat fetuses. The tissue was collected in a sterile 15-ml centrifuge tube containing 1 ml of phosphate-buffered saline (calcium- and magnesium-free) (Gibco 14200075). The tissue was enzymatically dissociated using 1 ml of 0.05% of trypsin-EDTA (Gibco 25300054) solution for 30 minutes in a 37°C water bath (100 rpm). After 30 minutes the trypsin solution was removed and 2 ml of L15 + 10% fetal calf serum (Gibco 16000044) was added to terminate the trypsin action. The tissue was then mechanically triturated. The supernatant was transferred to a 15-ml centrifuge tube. The same process was repeated two times by adding 2 ml of L15 + 10% FBS each time. The 6 ml cell suspension obtained after mechanical trituration was suspended on a 2 ml, 4% BSA (Sigma A3059) (prepared in L15 medium) cushion and centrifuged at 300 g for 10 minutes at 4°C. The pellet obtained was resuspended in 1 ml of serum-free medium and plated in 100 mm uncoated dishes for 30 minutes. The non-attached cells were removed, centrifuged on a 4% BSA cushion, and plated directly DETA coated glass covers slips or silicon cantilevers (controls). The cells were plated at a density of 700-1000 cells/$mm^2$. The cells attached to the substrate in 1 hour. The serum-free medium was added to the culture dish after 1 hour and the cells were maintained in a 5% $CO_2$ incubator (relative humidity 85%).

Motoneuron and DRG Preparation

Motoneuron and DRG cells were isolated from the dissected spinal cords of E14 rat embryos according to [3]. The spinal cords were subjected to an enzymatic dissociation in 0.05% trypsin (Invitrogen) then mechanical dissociation via trituration with a pasture pipette. The resulting mixture was layered on a metrizamide cushion in a 15 ml conical tube and centrifuged at 500 g for 15 minutes. The large cells remaining above the cushion were further selected to obtain motoneuron. Motoneurons were isolated by the immunopanning method using the immune interaction between motoneurons and the 192 antibody (1:2 dilution, ICN Biomedicals, Akron, OH) coated on 100 mm culture dishes. DRG cells were isolated from the pellet of the metrizamide cushion which essentially contains only DRG cells [3].

The resulting cellular isolates were resuspened in Neurobasal medium (Gibco-BRL) supplemented with B27 (2% v/v; Invitrogen), L-glutamine (0.5 mM), and 2-mercaptoethanol (25 μm). Three growth-promoting factors were added: glial-derived neurotrophic factor (100pg/ml GDNF; Invitrogen), brain-derived neurotrophic factor (100 pg/ml BDNF; Invitrogen) and ciliary neurotrophic factor (1 ng/ml CNTF; Cell Sciences). Cells were plated onto coverslips at 1.5-2.0 x $10^3$ cells/$mm^2$ for 1 hour. After 1 hour the serum-free medium was added to the culture dishes and the cells were maintained at 5% $CO_2$ at 37°C (85% relative humidity).

# 3 RESULTS

## 3.1 Surface Modification and Characterization

In the present study we used glass coverslips and silicon cantilevers coated with DETA. The modified surfaces were analyzed by contact angle and X-ray photoelectron spectroscopy (XPS). XPS has previously been shown to be a good quantitative indicator of monolayer formation [2,4-7]. The contact angle and XPS data indicated that the glass surfaces were covered by a complete monolayer of DETA.

The first step in creating this defined system was to develop a synthetic surface to control cell-substrate interactions. The current biological substrates (collagen, gelatin, fibronectin) offer little hope to create quantifiable cell-substrate interactions with systematic modifications [8]. It was also our objective that our surface modification method should be integratable with silicon cantilevers (Figure 1), compatible with surface patterning methods (stamping and photolithography) and it should enable relatively high throughput and flexible production of functionalized surfaces [2, 4]. Coating surfaces with self-assembled monolayers (SAMs) is a flexible and effective method to engineer surface characteristics of materials [9]. It has been shown that biological molecules can be incorporated into SAMs through crosslinkers, which could also enable selective study of specific contact signaling pathways [10]. SAM coated surfaces have also been used to grow and pattern hippocampal neurons, adult spinal cord neurons, motoneurons, cardiomyocytes, endothelial cells and muscle cell lines [2, 5, 10-14].

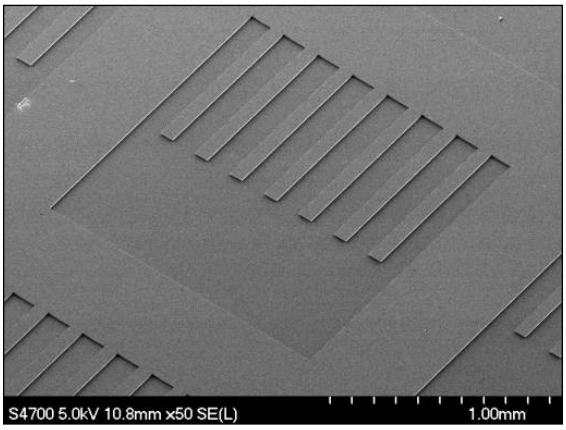


Figure 1: SEM of silicon cantilevers.

Our earlier experiments proved that DETA is an appropriate surface to grow and pattern neurons [2, 4, 6, 11, 14] and endothelial cells [5], and it is our hope to integrate the motoneuron and skeletal muscle culture by having a common surface modification.

The cell-attachment promoting feature of DETA is possibly a result of its hydrophilic properties and the presence of a primary amine group as indicated in earlier publications for neurons, cardiomyocytes and endothelial cells [2,4-7, 12, 14].

### 3.2 Serum-free Culture of reflex-arc elements on SAM modified microcantilevers and patterned surfaces.

The micrographs in Figure 2 (200x) show co-cultured motoneurons and primary muscle cells in serum-free media on SAM patterned surfaces. Shown on the left is a phase contrast micrograph of a motoneuron on a patterned DETA/13F surface contacting a developing muscle fiber. Shown on the right is a similar culture immunostained for neonatal a-actin (red) and neurofilament (green). These micrographs demonstrate both the adherence of an axonal process to the SAM pattern and the contact of that axon with a primary muscle cell or myotube. Both cultures are shown after 9 days in culture at 37°C, 5% $CO_2$, in L15 media. Similar results have been obtained for DRG cells on SAM modified surfaces (data not shown).

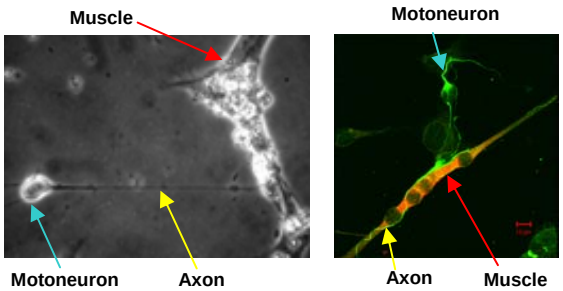


Figure 2: Co-cultures motoneurons and muscle on patterned surfaces.

In ten different experiments, we plated the dissociated muscle cells (obtained from E17 rat fetus) on unpatterned, DETA coated micro-cantilevers (Figure 3). In 50% of the cantilevers from each experiment, we observe that dissociated muscle cells aligned up along the long axis of the cantilever to form contracting myotubes. While the myotubes were observed to be contractile, it was not possible to visually confirm that they were bending the cantilevers. This is due to the fact that the spring constants of the cantilever were too high compared to the contractile strength of the myotubes. However, it cannot be said that the cantilevers did not bend at all, because no measurements sensitive enough to detect such a minute deflection were performed. These experiments have been reserved for future work. At this point we feel that integration of the myotubes with microstructures as indicated in Figure 3 is a significant step towards the future development of hybrid actuation systems for applications in biorobotics, prosthesis and bioartificial muscle engineering.

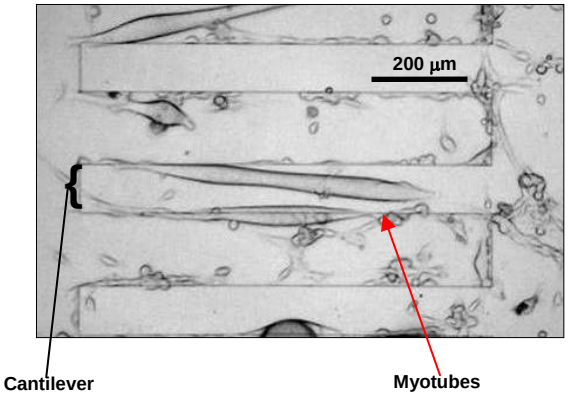


Figure 3: Cultured embryonic myotubes on cantilever.

## 4 DISCUSSION

In this work we have demonstrated the achievement of significant milestones in the development of a microCCA of the spinal reflex-arc for the study of SCI and neurodegenerative disease. We have demonstrated the ability to grow co-cultures of CNS neurons and muscle cells in a serum-free media. We have also shown that these co-cultured cells may form *in vitro* networks with geometries defined by variable surface chemistry. Data showing the integration of cellular elements and microstructures hints at the variety of other possible applications for microCCA systems. The development of a microCCA of the spinal reflex-arc will serve to improve our understanding of the basic biology and function of this fundamental unit of the nervous system. This system will combine the advantages of animal testing (presence of

complex physiological interactions and steady state environmental conditions) and *in vitro* testing (cost-effectiveness and rapid turnover). By creating a relatively inexpensive test bed that allows stringent control of culture conditions, real-time monitoring of cell/tissue properties, and high throughput data collection we could fundamentally change how basic cell biology and drug discovery are done

## REFERENCES


[1] Park TH, Shuler ML. Biotechnol Prog. 2003;19:243-253.

[2] Ravenscroft MS, Bateman KE, Shaffer KM, Schessler HM, Jung DR, Schneider TW, et al. J. Amer. Chem. Soc. 1998;120:12169.

[3] Das M, Molnar P, Devaraj H, Poeta M, Hickman J. Biotechnology Progress. 2003;19:1756.

[4] Schaffner AE, Barker JL, Stenger DA, Hickman JJ. J Neurosci Methods. 1995;62(1-2):111-9.

[5] Spargo BJ, Testoff MA, Nielsen TB, Stenger DA, Hickman JJ, Rudolph AS. Proc Natl Acad Sci U S A. 1994;91(23):11070-4.

[6] Stenger DA, Pike CJ, Hickman JJ, Cotman CW. Brain Res. 1993;630(1-2):136-47.

[7] Stenger DA, Georger JH, Dulcey CS, Hickman JJ, Rudolph AS, Nielsen TB, et al. J. Amer. Chem. Soc. 1992;114(22):8435-42.

[8] Daniels MP, Lowe BT, Shah S, Ma JX, Samuelson SJ, Lugo B, et al. Microsc Res Tech. 2000;49(1):26-37.

[9] Ulman A. Ultrathin Organic Films: from Langmuir-Blodgett to Self-Assembly. Boston: Academic Press; 1991.

[10] Mrksich, M. Chem Soc Rev. 2000;29(4):267-73.

[11] Acarturk TO, Peel MM, Petrosko P, LaFramboise W, Johnson PC, DiMilla PA. J Biomed Mater Res. 1999;44(4):355-70.

[12] Stenger DA, Hickman JJ, Bateman KE, Ravenscroft MS, Ma W, Pancrazio JJ, et al. J Neurosci Methods. 1998;82(2):167-73.

[13] Das M, Molnar P, Gregory C, Riedel L, Jamshidi A, Hickman JJ. Biomaterials. 2004;25(25):5643-7.

[14] Das M, Bhargava N, Gregory C, Riedel L, Molnar P, Hickman J. In Vitro Cell Dev Biol. 2005;41:343-348