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Abstract
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Disciplines
Chemical Engineering | Computer Engineering

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On-Chip Studies of Magnetic Stimulation Effect on Single Neural Cell Viability and Proliferation on Glass and Nanoporous Surfaces

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Abstract— Transcranial magnetic stimulation (TMS) is a non-invasive neuromodulation technique, an FDA-approved treatment method for various neurological disorders such as depressive disorder, Parkinson’s disease, post-traumatic stress disorder, and migraine. However, information concerning the molecular/cellular-level mechanisms of neurons under magnetic simulation (MS), particularly at the single neural cell level, is still lacking, resulting in very little knowledge of the effects of MS on neural cells. In this paper, the effects of MS on the behaviors of neural cell N27 at the single-cell level on coverslip glass substrate and anodic aluminum oxide (AAO) nanoporous substrate are reported for the first time. First, it has been found that the MS has a negligible cytotoxic effect on N27 cells. Second, MS decreases nuclear localization of paxillin, a focal adhesion protein that is known to enter the nucleus and modulate transcription. Third, the effect of MS on N27 cells can be clearly observed over 24 hours—the duration of one cell cycle—after MS is applied to the cells. The size of cells under MS was found to be statistically smaller than that of cells without MS after one cell cycle. Furthermore, directly monitoring cell division process in the microholders on a chip revealed that the cells under MS generated statistically more daughter cells in one cell cycle than those without MS. All these results indicate that MS can affect the behavior of N27 cells, promoting their proliferation and regeneration.

Index Terms— neural cells, nanoporous surface, microholder chip, magnetic stimulation (MS), neural cell proliferation and regeneration.

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INTRODUCTION

Transcranial magnetic stimulation (TMS) is a non-invasive neuromodulation technique that uses time-varying short pulses of magnetic fields to induce an electric field in the conductive tissues of the brain, thus modulating the synaptic transmission of neurons. This neuromodulation technique can be used to excite or inhibit the firing rate of neurons as a treatment for various neurological disorders such as major depressive disorder, Parkinson’s disease, post-traumatic stress disorder, and migraine\textsuperscript{1-2}. While the effect of magnetic stimulation (MS) on other types of cells and biomolecules \textit{in vitro} has been studied in the past\textsuperscript{3-7}, information concerning the molecular/cellular mechanisms of neurons under MS, particularly at the single neural cell level, is still lacking. The effects of MS on single neural cells need to be thoroughly understood in order for experts to make the greatest use of MS as a neuromodulation tool for treating neurological disorders, especially those originating from the subcortical regions of the brain.

In order to study the behaviors of single neural cells \textit{in vitro}, a solid substrate or scaffold is usually needed for the cell’s proper adhesion, spreading, and growth\textsuperscript{8}. It is particularly desirable to create a substrate or scaffold mimicking the native extracellular environment that can interface with individual neural cells within living tissues and in cell culture. As previous studies have demonstrated, substrates or scaffolds with nanoscale features (i.e. nanostructured bio-interfaces) have greatly improved specificity and accuracy for many neural-engineering applications\textsuperscript{9-11}, including neural probes for Parkinson’s patients and guidance scaffolds for axonal regeneration in patients with traumatic nerve injuries\textsuperscript{12-13}, just to name a few.

Hence, nanostructured bio-interfaces have become a rapidly emerging area of study. For instance, over the past decades, which have witnessed the development of nanotechnology and nanomaterial that is safe for biological applications, interfaces between a variety of nanomaterials or nanostructures with biomolecules have been studied\textsuperscript{14}. Examples of nano-bio-interfaces include the interface of the nanoparticle-lipid bilayer\textsuperscript{15}, the interface of carbon nanotubes (CNTs)-biomolecules\textsuperscript{16}, and the interface
of graphene-biomolecules. The studies of nano–bio-interfaces primarily aim to understand the dynamic physicochemical interactions, kinetics, and thermodynamics between nanomaterial surfaces and the surfaces of biological components, which include, for example, proteins, membranes, phospholipids, endocytic vesicles, organelles, DNA, and biological fluids. One widely used nanostructured material is anodic aluminum oxide (AAO). The unique properties of nanoporous AAO thin film have greatly contributed to the development of a variety of novel biomedical and medical applications, ranging from biofiltration membranes, lipid bilayer support structures, biosensing devices, and implant coatings to drug delivery systems with AAO capsules and scaffolds for tissue engineering. Additionally, nanoporous AAO membranes have generated increasing interest and shown great promise as cell-interface substrates for manifold cell types.

Parkinson’s disease (PD), the second most common neurodegenerative disorder after Alzheimer’s disease (AD), is caused by the death of dopaminergic neurons in the substantia nigra. N27 neural cells have been widely used for their dopaminergic properties as an in vitro model of PD. In addition, they have been extensively used for studies of neurotoxicity, oxidative stress, neurodegeneration, and other molecular pathways. The effects of MS on their functions are currently unknown.

Previously, we have found the MS stimulates N27 cells proliferation in a population-based assay. However, we did not analyze single cells behavior, nor did we assess other cell functions that modulate proliferation like cytoskeletal structure or adhesion. In this paper, an AAO nanoporous surface is used as one of the substrates to study the behavior of N27 neural cells under MS. The effects of MS on the single-cell level spreading, cytoskeletal structure, adhesion and proliferation on a glass surface and an AAO nanoporous surface are reported for the first time.
MATERIALS AND METHODS

A. Materials

(1) The experiments use neural cell N27 (Millipore Sigma), which stands for immortalized rat mesencephalic cells (1RB3AN27). The following chemicals are used for culturing the N27 neural cell: RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich); 1% L-glutamine (Sigma-Aldrich); penicillin with a concentration of 100 U/ml (Sigma-Aldrich); and streptomycin in 100 U/ml (Sigma-Aldrich). (2) Fluorophores, calcein AM (Sigma-Aldrich), and propidium iodide (Sigma-Aldrich) are used for monitoring cell viability using a fluorescence microscope (Olympus, Inc.). (3) Scanning Electron Microscopy (SEM) is used to image the neuron cell growth on different substrates. Standard PBS (Sigma-Aldrich), 4% paraformaldehyde in a 0.1 M PO4 buffer (Sigma-Aldrich), Ethanol >99% (Sigma-Aldrich), and Hexamethyldisilazane (HMDS) >99 % (Sigma-Aldrich) are used for sample pretreatment before SEM imaging. (4) Aluminum pellets with a purity of 99.999% (Iamaterials, Inc.), Titanium pellets with a purity of 99.995% (Iamaterials, Inc.), and SU-8 3025 (Microchem, Inc.) are used to fabricate microholder chips.

B. Substrates for the studies of viability and behaviors of neuron cells N27

Different surfaces/substrates were prepared for studying neuron cell growth: standard coverslip glass (Thermo Fisher Scientific, Inc.), AAO substrates fabricated by a one-step anodization process, and AAO substrates fabricated by a two-step anodization process. The procedure for preparing the AAO substrates is described in detail in our previous work\textsuperscript{31}. Briefly, after a layer of Al is deposited on a glass substrate, a one-step anodization process is carried out to convert the Al into a nanoporous AAO thin film. An SEM image of the AAO substrates fabricated by the one-step anodization process is shown in Fig. 1a. The two-step anodization process begins with 10 minutes of anodizing the Al layer in 0.3 M oxalic acid. Then the samples are etched with a mixture of phosphoric acid (0.4 M) and chromic acid (0.2 M) at 65 °C for
30 minutes, followed by a 40-minute step-two anodization in 0.3 M oxalic acid with the same experimental condition as the step-one anodization. An SEM image of the AAO substrates fabricated by the two-step anodization process is shown in Fig. 1b. The average nanopore diameter of the one-step AAO substrates (AAO-substrate1) is ~20 nm, and its average porosity is 6.6%. The two-step AAO substrates (AAO-substrate2) have an average diameter of ~80 nm and an average porosity of 84.11%. As shown, the nanopore size and porosity of the AAO-substrate2 are larger and more uniform than those of AAO-substrate1. The two AAO substrates therefore have differently nanostructured topological surfaces.

C. Arrayed SU8 microholder chip for studying the viability and growth of single neural cell N27

Two types of SU8 microholder chips have been designed and fabricated. One type consists of arrayed microholders embedded with glass surfaces; the other type consists of arrayed microholders embedded with nanoporous AAO surfaces. The fabrication processes flow for the SU8 microholder chip with an AAO nanoporous surface (AAO microchip) and for the SU8 microholder chip with a glass surface (glass microchip) are described in Fig. S1 (in the supplementary document). Fig. 1c shows a photo of an SU8 microholder chip and the close-up optical micrographs of the microholders.

D. Substrate cleaning, cell synchronization, cell seeding, and SEM imaging

Sterilization: Both AAO nanoporous substrates and glass substrates are cleaned for 5 minutes with DI water using the ultra-sonicator (BRANSON 1510-Fisher Scientific), and sterilization of these substrates is achieved by cleaning them with acetone and methanol with the ultra-sonicator for 5 minutes in sequence. The substrates are then cleaned with DI water by ultra-sonicating for 5 minutes and dried by a nitrogen gun, followed by air plasma cleaning (Harrick plasma-PCD 32G) for 3 minutes. Finally, they are baked for 5 minutes on a hot plate at 150 °C before use.

Cell synchronization: In general, cells in a culture will exist in different stages within the cell cycle at any one moment. The biospecific differences of each cell make it difficult to remain synchronized over long
times\textsuperscript{32}. In order to obtain statistically accurate results of the effect of MS on the cell division process for a large number of cells, cell synchronization, a process that brings cells at different stages of the cell cycle to the same phase, must be implemented. To this end, a simple serum deprivation method is used by changing the percentage of fetal bovine serum from 10\% to 1\% in the cell culture medium\textsuperscript{32-33}. Specifically, the medium of 10\% fetal bovine serum is replaced with 1\% fetal bovine serum 9 hours before the cells are seeded into the microholders. A 9-hour serum deprivation time was chosen for this study for a number of reasons. First, cell mortality was taken into account. Since the serum contains essential components for cell growth, cell death increases with the serum deprivation time. Based on our experiments, a 9-hour serum deprivation does not cause substantial cell death. This 9-hour synchronization period ensures that most of the cells have been synchronized at the G1 phase, the first of four phases of the cell cycle that takes place in eukaryotic cell division\textsuperscript{33}.

**Cell seeding:** Trypsin is used to detach the cells from the bottom of the flask before the cells are seeded on different substrates. After 2 or 3 minutes, we pipette a normal medium with 10% fetal bovine serum to the flask. Then the cells are ready to be seeded to the microholder devices.

**Fixing and staining cells:** Cells are plated on either AAO-substrate2 or glass microscope slides in 10% fetal bovine serum media as described above and fixed and stained for F-actin and paxillin with the following protocol. Cells are fixed in 4\% paraformaldehyde (\textit{Fisher Scientific}) w/v dissolved in cytoskeleton buffer at 37 °C for 15 minutes. Cytoskeleton buffer is a solution containing 10 mM MES (\textit{Sigma Aldrich}), 3 mM MgCl\textsubscript{2} (\textit{Fisher Scientific}), 138 mM KCl (\textit{Fisher Scientific}), and 2 mM ethylene glycol-bis (β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA) (\textit{Sigma Aldrich}) at pH = 6.1. Cells are permeabilized with a 0.5% triton X solution for 5 minutes. The samples are then treated with 0.1 M glycine (\textit{Fisher Scientific}) for 10 min. Both glycine and triton X solutions are prepared in the cytoskeleton buffer. After glycine treatment, cells are washed with Tris-buffered saline (TBS) containing 150 mM NaCl (\textit{Fisher Scientific}) and 20 mM Tris-Cl (\textit{Fisher Scientific}) at pH = 7.4 multiple times.
Blocking and F-actin staining is conducted simultaneously. The blocking buffer is made with TBS, 0.1% v/v tween-20 (Fisher Scientific), and 2% w/v bovine serum albumin (BSA) (Sigma Aldrich). A 1:200 dilution of alexa 488-phalloidin (Molecular Probes) was added to the blocking buffer. The samples are incubated for one hour at room temperature and are protected from light. After incubation, the cells are washed in TBS multiple times. Cells are then incubated with purified mouse anti-paxillin antibody (BD Transduction Laboratories, clone 349) in blocking buffer at 1:200 ratio for an hour at room temperature and protected from light, followed by washing in TBS multiple times. Then the cells are incubated with donkey anti-mouse Cy3 conjugated antibody (Jackson Immuno Research Laboratories) in blocking buffer at 1:200 ratio for an hour at room temperature and light protected conditions. Further, the samples are washed in TBS multiple times. Mounting media solution consists of 75% v/v glycerol and 20 mM Tris solution at pH = 8. The samples are inverted onto mounting media on number 1.5 coverslips and sealed with nail polish.

**Imaging cells:** Fluorescence imaging is conducted on a fluorescence microscope (Olympus, Inc.) which includes the following filter sets: FITC (excitation filter: 460-495 nm; barrier filter: 500-540 nm); and TRITC (excitation filter: 545-565 nm; barrier filter: 580-620 nm). Images are captured through a 40 × objective. Exposure times are kept constant for a particular fluorophore (alexa 488 or Cy3). Twenty images from each condition are captured.

**Focal adhesion analysis:** Analysis is conducted using ImageJ (National Institutes of Health). The F-actin images are thresholded and used to specify the outline of the cell for both F-actin and paxillin images. The paxillin image is thresholded differently to specify the outline of the nucleus. Whole cell F-actin and paxillin and nuclear average intensity is background subtracted. Cytosolic paxillin intensity is calculated from the integrated whole cell paxillin intensity less the integrated nuclear intensity divided by the whole cell area less the nuclear area. Peripheral and central focal adhesions are manually counted. Binucleated cells are identified as nuclei that could not be segmented using a threshold to find the nuclear outline.
Cell labeling with fluorophores and fluorescence imaging for live/dead assay: In order to carry out the live/dead assay and monitor cell viability, we label the cells with fluorophores calcein AM and propidium iodide, using a concentration of 2 µM. Fluorescence images are captured with a fluorescence microscope (Olympus, Inc.).

SEM imaging: The following steps are taken to obtain SEM images of the cells’ growth on the glass and AAO surfaces. First, after 3 days of growth, the samples are rinsed with PBS. Then the neuron cells are fixed in 4% paraformaldehyde resolved in a 0.1 M phosphate (PO₄) buffer with a pH of 7.4 for 20 minutes at room temperature. Second, the samples are rinsed 5 times with PBS at room temperature and then with a DI water rinse for 10 minutes. Third, dehydration is achieved by immersing the samples into solutions of 25 %, 50 %, 70 %, 80 %, 85 %, 90 %, 95 %, and 100 % ethanol for 10 minutes each, followed by an additional two rinses with 100% ethanol. Last, the samples are rinsed with three mixtures of ethanol and HMDS, with volume ratios of 3:1, 1:1, and 1:3, for 10 minutes each. Then the samples are rinsed twice with pure HMDS for 10 minutes and left in the HMDS until completely dry. The samples are then ready for SEM imaging.

E. Experimental setup for applying MS to N27 neural cells

The Magstim 2002 monophasic stimulator with an ‘8’-shaped coil (Magstim D702 double 70 mm) is used to apply MS to the cells, as shown in Fig. S2a (in the supplementary document). The magnetic fields are generated in opposite directions through the two windings and are perpendicular to the surface of the ‘8’-shaped coil so as to be perpendicular to the chip holder on both sides. As shown in Fig. S2b, an upward magnetic field (MF) is generated from the left side of coil, and a downward MF is introduced from the right. The power level is set to 100 % and the MF strength for the coil is shown in Fig. S2c. The upward peak MF strength is ~1.5 MA/m and the downward peak MF is ~1.25 MA/m.
E. Statistical analysis

The study focuses on cell growth under two different experimental conditions. First, the cell morphology (i.e. area) of each single N27 cell on different substrates after a 48-hour incubation period has been measured. Second, hundreds of single N27 cells have been captured inside each SU8 microholder, and the division process of each cell has been monitored by counting cell numbers in each SU8 microholder after a 48-hour incubation period. For each case, several trials have been conducted to collect sufficient data for statistical analysis. The experimental conditions and parameters are summarized in Table 1.

Specifically, for the morphometric analysis of N27 cells, experiments with N27 cells on three substrates (AAO-substrate1, AAO-substrate2, and glass substrate) have been carried out. For all experiments, MS is applied when the cells have been seeded and grow on the substrates 24 hours later. In other words, the cells settle on the substrate’s surface and are allowed one day of growth before the application of MS. Three independent trials (NT = number of trial) have been performed for each substrate. For each trial, at least 65 cells (NC = number of cell) are selected for cell area measurement. More details can be found in Table S1 (in the supplementary document).

In order to analyze the N27 cell division process, experiments on two types of microchip (AAO and glass) are performed. On both microchips, only microholders with single N27 cell captured in each are monitored in order to track cell division accurately. For both AAO and glass microchips, MS is only applied after one-day (24-hour) growth of the captured cells. Five independent trials are performed for each substrate. For each trial, at least 150 single N27 cells are captured in 150 microholders. More details can be found in Table S2 (in the supplementary document).

SPSS statistical analysis is used to analyze the experimental data. Tests of normality, outliers, and homogeneity variance are performed for each group of data before a one-way analysis of variance (ANOVA) \(^{34}\). The statistical significance is reported with \(p < 0.05\) and \(p < 0.005\) for each group of data.
The one-way ANOVA test results are shown in the following section, and more detailed information about the statistical analysis can be found in the supplementary material.

RESULTS AND DISCUSSION

A. Spreading of N27 cells on different substrates

The N27 cells are seeded on an AAO nanoporous surface (Fig. 2a-b) and on a coverslip glass surface (Fig. 2f-g). Cells are then cultured in an RPMI medium supplemented with 10 % fetal bovine serum, 1 % L-glutamine, penicillin (100 U/ml), and streptomycin (100 U/ml). Petri dishes are used as the container, and all the samples are kept in an incubator (NUAIRE-5700) at 37 °C in a humidified atmosphere of 5 % CO₂ during the cell culture process. It should be noted that both the coverslip glass surface and the AAO nanopore surface shown in Fig. 2 are bare surfaces used directly after the cleaning process without any further surface treatment.

As shown in Fig. 2a-b, cells spread out clearly on the AAO nanoporous surface over the course of 24 hours of growth and 3 days of growth alike. And as shown in Fig. S3, cells can also spread out clearly on the common cell culture flask, similar to those on the AAO nanoporous surface. By contrast, “ball-shaped” cells are observed after one day of growth on coverslip glass (Fig. 2f). After three days of growth, over 50 % of these cells still grow in clusters (Fig. 2g), indicating that they have a relatively slower migration than those on the AAO nanoporous substrate 35-37. AFM images of the AAO substrate and glass substrate are shown in Fig. 2c and Fig. 2h, respectively. As indicated, the surface roughness of the AAO substrate is significantly greater than that of the coverslip glass. Clearly, the nanoporous surface of the AAO substrate is much preferred for cell adhesion and spreading 38-40. Thus, the morphology has obviously been improved compared to that of the cells on coverslip glass 41. The corresponding SEM images of a single N27 neural cell on the AAO surface and on the glass surface are shown in Fig. 2d-e and Fig. 2i-j, respectively. The nanopore structures on the AAO surface can facilitate the attachment,
adhesion and spread for N27 cells compared to the smooth surface of coverslip glass \(^{38-40}\). It has also been reported that the cell adhesion does play an important role in cell proliferation of cancer cells based on biochemical analysis \(^{42}\).

In order to characterize the difference in adhesion shown on AAO and glass substrates, we fixed and stained for both F-actin and paxillin in N27 cells (Fig. 3a). As suggested in Fig. 2, cells on AAO substrates were frequently separated from each other as compared to glass. On glass, cells clustered together. This is frequently seen when cell migration is slow compared to cell proliferation \(^{43}\). In addition, cells on AAO surface appeared to be better spread (Fig. 3a). The formation of F-actin as well as focal adhesions occur during adhesion, so we measured the levels of F-actin and paxillin, a protein that is localized to focal adhesions \(^{44-45}\) (Fig. 3a). At 24 hours after plating there was a small enhancement in F-actin content in N27 cells plated on glass substrate vs. AAO substrate (Fig. 3b). This difference was eliminated at 48 hours (Fig. 3c). Interestingly, there appeared to be a significant paxillin signal coming from the nucleus in addition to that seen in focal adhesions. (Fig. 3a) Paxillin is known to shuttle into the nucleus to affect gene transcription that leads to proliferation \(^{46-47}\). Enhanced shuttling to the nucleus appears to be due to turnover of paxillin in focal adhesions \(^{48}\). The nuclear paxillin signal was the same at 24 hours after plating on AAO or glass substrates. (Fig. 3b) However, at 48 hours, the paxillin in the nucleus was significantly higher in cells plated on AAO substrates than cells plated on glass (Fig. 3c). The opposite trend was seen for cytosolic paxillin, which was higher at both 24 and 48 hours on glass as it was on AAO substrates. This suggests more mature adhesions related to less motile cells. In addition to the enhanced paxillin localization in the nucleus, cells plated on AAO substrates had a lower number of central focal adhesions as compared to peripheral focal adhesions (Fig. 3d). Furthermore, central focal adhesion numbers increased with respect to peripheral focal adhesion numbers and the plating time increased (Fig. 3d). Central focal adhesions are a marker for less motile cells and tend to turnover slower \(^{39-40}\). The absence of these central focal adhesions potentially explains the higher paxillin localization in
the nucleus of the cell on AAO substrates. Indeed, others have shown similar focal adhesion characteristics on AAO substrates compared to glass \(^{39-40}\). Finally, when cells are highly adherent and lack motility, they tend to produce cytokinesis defects on 2D substrates, resulting in binucleated cells (Fig 3e). Taken together, an AAO substrate appears to better promote migration than a glass substrate resulting in non-clustered cells. The enhanced migration on AAO substrates is caused by the ability of focal adhesions to turnover quicker, leading to more paxillin in the nucleus. On glass substrates, migration is blocked and central and stable focal adhesions are formed, which act to inhibit the accumulation of paxillin in the nucleus as well as block proper cytokinesis of cells, resulting in more binucleated cells.

**B. Viability of N27 cells on different substrates under MS**

In order to assess the viability of N27 cells under MS, cellular viability tests by calcein AM and propidium iodide (PI)-based live/dead assay have been performed \(^{49}\). In these experiments, four substrates have been used, including coverslip glass, AAO substrate, glass microchip, and AAO microchip. All the surfaces of these substrates are coated with poly-D-lysine. MS is applied after the cells grow on these substrates for 24 hours (i.e. one cell cycle). As expected \(^{50}\), the cell morphology on the poly-D-lysine-coated coverslip glass in Fig. 4a, c is superior to that on bare coverslip glass in Fig. 2f, g and closer to that on bare AAO substrate in Fig. 2a, b and poly-D-lysine-coated AAO substrate in Fig. 4b, d. As evident from the fluorescence images (Fig. 4a-d) and viability quantification of the cells (Fig. 4e) on the four substrates, MS has a negligible cytotoxic effect on cells. Furthermore, as shown in Fig. 4c-d, cell division can also be observed clearly. For example, in the image from the glass microchip in Fig. 4c, a single N27 cell is captured and divided into two daughter cells 24 hours (one cycle) later. At this point, MS is applied, and the two daughter cells grow and are divided into five live cells 24 hours later (another cell cycle). Similarly, on the AAO microchip seen in Fig. 4d, a single N27 cell is divided into two
daughter cells 24 hours later without MS. At this point, MS is applied, and the two daughter cells grow
and are divided into six daughter cells after another 24 hours.

C. MS effects on N27 neural cell growth

(1) MS effects on morphology (area) of N27 cells on glass and AAO substrates

In these experiments, both poly-D-lysine-coated glass and poly-D-lysine-coated AAO substrates are
used. Representative images showing the area size of the N27 cells, measured by an accessory imaging
analysis software provided with the fluorescence microscope (Olympus, Inc.) are given in Fig. 5a.

First, statistical comparisons are conducted of cell morphology (area) on the glass substrate, AAO-
substrate1, and AAO-substrate2, all without the administration of MS. In other words, these
comparisons only measure the effect of each substrate’s topological surface on cell morphology. After
48 hours of incubation, the area of each single neuron cell is measured, shown in Fig. 5b(1). The area of
the cell on AAO-substrate1 (~538 µm²) is smaller than that on AAO-substrate2 (~551 µm²). By contrast,
the cell area on the glass substrate (~582 µm²) is larger than those on AAO-substrate1 and AAO-
substrate2. Detailed statistical analysis can be found in the supplementary document. In short, the N27
cell morphology on both AAO substrates is statistically different from that on the glass substrate.
However, there is no statistically significant difference in the N27 cell morphology on AAO-substrate1
in comparison to that on the AAO-substrate2, suggesting that differences in nanopore size and porosity
among AAO substrates have no clear effect on cell growth.

Second, the cell areas on the three substrates (AAO-substrate1, AAO-substrate2, and glass) with and
without MS are shown in Fig. 5b(2)-(4). The area of the cell without MS on AAO-substrate1 (~538
µm²) is larger than that (~520 µm²) with MS. Similarly, on AAO-substrate2, the cell area without MS
(~551 µm²) is larger than that with MS (~537 µm²). In terms of the glass substrates, the cell area without
MS (~582 µm²) is also larger than that with MS (~519 µm²). Detailed statistical analysis can be found in
the supplementary document. All the results indicate that MS indeed affects cell spreading. Specifically, for all three substrates, the area of the cells that received MS is statistically smaller than that of the ones that did not receive MS. It is important to note that MS is applied to the cells after 24 hours of growth, or one cell cycle, and that the measurements are taken 24 hours later.

(2) MS effects on the cytoskeleton and paxillin localization

In addition to spreading area, we analyzed the F-actin content as well as paxillin localization in cells with and without MS. While the substrate type (glass vs. AAO substrates) did affect cell morphology, there were no gross changes in the cell morphology after MS (Fig. 3a & Fig. 6a). In addition, there were no large differences in the cytosolic paxillin fluorescence comparing with and without MS (Fig. 6b). Interestingly, the paxillin level in the nucleus, which was high on AAO substrates decreases when the cells were treated with MS and could be associated with changes in transcription brought on by MS (Fig. 6b). This was not seen on glass and may only be observable in well-spread cells. MS did not seem to change the central to peripheral focal adhesion ratio on AAO substrates, but it did decrease it on glass (Fig. 6c). Finally, there is a small increase in the number of binucleated cells after MS on AAO substrates (Fig. 6d). Taken together, it appears that MS decreases paxillin localization to the nucleus. This likely leads to or is caused by enhanced adhesion, resulting in cytokinesis defects leading to binucleated cells.

(3) MS effects on single N27 neural cell on microchip

In order to assess the effect of MS on cells more efficiently, arrayed microholder chips have been used to monitor the division process of single cells subject to MS. First, cell synchronization is needed to ensure the cells are at the same stage of the cell cycle, which can be achieved using the serum deprivation method described in the section of Materials and Methods 32-33. Second, in order to monitor the cell division process accurately, one single N27 cell is captured in each microholder, enabling its division
process to be tracked over the following days. To this end, several steps are required to maximize the
capture of the single cell into each microholder: (1) After cells are detached from the bottom of the cell
culture flask, they are pipetted into a sterile culture tube (Corning) that is put on a vortex mixer (VX200-
Labnet) with a rotation speed of 500 rpm. This step ensures that the cells are uniformly distributed in the
tube and that there are no aggregated cells. (2) The concentration of cells is controlled close to 0.1
million/ml. Based on our experiments, 0.1 million/ml is the optimum cell concentration to maximize the
number of microholders on-chip to capture a single cell when the cell solution is delivered to the chip by
pipette. (3) The chip is given several gentle rinses with the culture medium (10 % fetal bovine serum) to
achieve optimum single cell capture.

Accordingly, single cell division in a microholder is monitored by taking optical images at 0 hours, 24
hours, and 48 hours, as shown in Fig. 7. Fig. 7a shows that, when MS is not administered, a cell typically
divides into two daughter cells after one cell cycle (~24 hours) and into four cells after another cell cycle
(~48 hours in total). However, when MS is applied at the 24-hour mark, the two cells divide into more
than six cells after another cell cycle (~48 hours in total), as shown in Fig. 7b. As a result of MS, more
than two new daughter cells manifest within the same period of time compared to the chip with cells that
do not receive MS (Fig. 7a). Clearly, the microholder chip provides a straightforward technical platform
to conduct an accurate analysis of the effect of MS on cell division.

Several trials have been done to study the MS effect. For each trial, three microchips with captured
cells are immersed in cell media in three Petri dishes and are stored in an incubator (NUAIRE-5700) at all
times save for when the MS is applied. Upward MS and downward MS are applied on two microchips,
respectively, the third microchip serving as a control without any MS. The experimental details and
analysis are summarized in Table S2 (in the supplementary document).

First, statistical comparisons of cell division are conducted on the glass microchip and the AAO
microchip without applying MS. Since no MS is applied to these cells, any differences in outcome are
due solely to the surface properties of the glass and AAO surfaces. As shown in Fig. 7c(1), the average number of cells (~4.08) on the glass microchip is smaller than that (~4.28) on the AAO microchip, a statistically significant difference. Detailed statistical analysis can be found in the supplementary document. In short, the nanoporous surface of the AAO microchip affects N27 cell growth, promoting the cell division process in comparison with the flat surface of the glass microchip.

Second, when upward-MS is applied to cells on a glass microchip after 24 hours of growth, as shown in Fig. 7c(2), the average cell number after a further 24 hours (~4.87) is greater than the cell number of the control group that received no MS (~3.87). Similarly, when downward-MS is administered to cells on a glass microchip, the average cell number at the 48-hour mark (~4.64) is larger than that of the control. Detailed statistical analysis can be found in the supplementary document. Clearly, the average cell number without applying MS is statistically smaller than that with MS. This result indicates that MS can promote cell division. However, no statistical difference has been found between upward-MS and downward-MS, suggesting that the direction of MS has no effect on the cell division process in these contexts.

Third, when MS is applied to cells on an AAO microchip after a 24-hour period of growth, as shown in Fig. 7c(3), the average cell number at the 48-hour mark is ~4.73 with upward-MS and ~4.85 with downward-MS. By contrast, the average cell number of the control group receiving no MS is ~4.18. These constitute similar results to those observed on the glass microchip. In both cases, the average number of cells is increased when MS is administered after 24 hours (i.e. one cell cycle) of growth, indicating that the proliferation of N27 cells can be expedited by MS. The MS direction has no effect on cell division.

Several important elements in the experiments should be noted. First, if the MS is applied immediately after the cells are seeded on the substrates or microchips, the experimental results are not consistent, and the viability of the cells is unpredictable. Allowing the cells to attach to the substrate and allowing them
to grow for one day before applying MS are crucial steps to ensure consistent experimental results. Hence, we only focus on the experiments in which MS is applied to cells after cells have been given one full cycle of growth (24 hours) on the substrate. Second, the effect of MS on cells can be clearly and immediately observed in one cell cycle (i.e. 24 hours); hence we mainly focus on monitoring cells’ behaviors during a 24-hour period after administration of MS. Notably, experiments that have monitored cell growth 72 hours and longer after MS have consistently shown increased cell numbers in comparison with control groups.

Conclusions

This study constitutes the first investigation into the effect of MS on the viability, spreading and proliferation of N27 cells at the single-cell level on different substrates. N27 cells have been found to have clear preference for growing on the bare nanostructured AAO surface as opposed to the bare coverslip glass surface. The nanostructures on the AAO surface allow the N27 cells to attach, spread, and migrate easily. The AAO surface appears to facilitate paxillin movement into the nucleus as well as limit central adhesions that inhibit or are the result of poor migration. A poly-D-lysine coating on either of these surfaces leads to no discernible difference in the spreading of the cells. A live/dead assay has shown negligible cytotoxic effects of MS on cell viability. Additionally, the study has evaluated the effect of MS on the adhesion and proliferation of single N27 cells by measuring cell sizes and monitoring the cell division process in the microholders on a chip. MS has been found to have a statistically significant effect on both cell morphology and cell division/proliferation and it results in lower paxillin levels in the nucleus. Specifically, MS can expedite the division of N27 cells regardless of the direction in which it is applied. As a result, more N27 cells can be generated with MS than without. These results indicate that MS may help the proliferation and regeneration of neural cells; more largely, the results enhance current understanding of the potential for MS to be used in the treatment of neurological disorders.
AUTHOR DISCLOSURE STATEMENT

No competing financial interest exists.

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Supporting Information. The process flow for fabricating microchips; the control experiments for N27 cells’ growth on the cell culture flask; and the detailed statistics analysis.
Table 1: Summary: experimental conditions and parameters

<table>
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<th>Experiments</th>
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<th>NC (# of cells)</th>
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<tr>
<td></td>
<td>AAO-substrate2</td>
<td>24 hrs</td>
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<tr>
<td></td>
<td>Glass substrate</td>
<td>24 hrs</td>
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<td>196</td>
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<td>Cell division (cell count)</td>
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<td>880</td>
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<tr>
<td></td>
<td>AAO microchip</td>
<td>24 hrs</td>
<td>5</td>
<td>2073</td>
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Figure 1: SEM images of (a) AAO-substrate1: nanopore diameter = 20 nm, porosity = 6.6%; (b) AAO-substrate2: nanopore diameter = 100 nm, porosity = 84.11%; (c) A photo of microholders on chip and the close-up optical micrographs of arrayed SU8 microholders and the captured single neural cell N27.
Figure 2: N27 neural cell growth on nanoporous AAO-substrate (a-e) vs glass coverslip substrate (f-j): (a) and (f) after one-day growth; (b) and (g) after three-day growth (cells are indicated with red arrows); (c) and (h) AFM images of AAO surface and glass surface, respectively; (d-e) and (i-j) SEM images for a single N27 neural cell growing on AAO surface and glass surface, respectively.
Figure 3: F-actin and paxillin staining of N27 neural cells on nanoporous AAO-substrate2 and glass (a) Cells stained for F-actin and paxillin after 24 hrs. and 48 hrs. on AAO-substrate2 and glass. The scale bar is 40 µm. Whole cell F-actin as well as nuclear paxillin and cytosolic paxillin staining after (b) 24 hrs. and (c) 48 hrs.; (d) number of center to peripheral focal adhesions as identified by paxillin staining; (e) number of binucleated cells. Error bars are 95% confidence intervals and non-overlapping confidence intervals were considered statistically significant and marked with bars.
Figure 4: Effect of MS on N27 cell viability. Live/dead assay on N27 cells cultured on (a) coverslip glass, inset is the close up image of N27 cells, (b) AAO-substrate2, inset is the close up image of N27 cells, (c) glass microchip (cells are indicated with red arrows), (d) AAO-substrate2 microchip (cells are indicated with red arrows). Green (calcein AM) and red (propidium iodide: PI) denote live cell and dead cell, respectively. MS is applied at 24 hrs; (e) % viability of N27 cells cultured on four substrates for 24 hrs (blue) and 48 hrs (orange), respectively. NS denotes no statistically significant difference.
Figure 5: (a) Representative images showing N27 cells grown on a nanoporous AAO-substrate1; their sizes (areas) are measured by an imaging analysis software; (b) quantification of cell size of N27 cells cultured on three different substrates without or with MS at 24 hrs. Asterisks * and ** denote statistically significant differences with $p < 0.05$ and $p < 0.005$, respectively. NS denotes no statistically significant difference.
Figure 6: F-actin and paxillin staining of N27 neural cells on nanoporous AAO-substrate2 after magnetic stimulation (+MS) (a) Cells stained for F-actin and paxillin after 48 hrs. on AAO substrate2 and glass and with or without MS at 24 hrs. The scale bar is 40 µm; (b) Whole cell F-actin as well as nuclear paxillin and cytosolic paxillin staining after 48 hrs. on AAO or glass substrates with and without MS; (c) number of center to peripheral focal adhesions as identified by paxillin staining; (d) number of binucleated cells. Error bars are 95% confidence intervals and non-overlapping confidence intervals were considered statistically significant and marked with bars.
Figure 7: Representative images showing (a) the division of a single neuron cell in a microholder without TMS, (b) the division of a single cell with MS. The cells are indicated with red arrows. (c) Quantification of cell number at 48 hrs in glass microchip and AAO-substrate2 microchip without or with TMS at 24 hrs. Asterisks * and ** denote statistically significant differences, with p < 0.05 and p < 0.005, respectively. NS denotes no statistically significant difference.
Reference:


TOC Graphic