Passive pumping for the parallel trapping of single neurons onto a microsieve electrode array

Citation for published version (APA):

DOI:
10.1116/1.4991827

Document status and date:
Published: 01/11/2017

Document Version:
Publisher’s PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:
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Citation: Journal of Vacuum Science & Technology B, Nanotechnology and Microelectronics: Materials, Processing, Measurement, and Phenomena 35, 06GA01 (2017);
View online: https://doi.org/10.1116/1.4991827
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Passive pumping for the parallel trapping of single neurons onto a microsieve electrode array

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(Received 23 June 2017; accepted 8 November 2017; published 27 November 2017)

Recent advances in brain-on-a-chip technology have led to the development of modified microelectrode arrays. Previously, the authors have contributed to this exciting field of neuroscience by demonstrating a fabrication process for producing microsieve chips that contain three-dimensional (3D) micropores at the electrodes [termed microsieve electrode arrays (μSEAs)]. This chip allows us to trap hundreds of single neuronal cells in parallel onto the electrodes [B. Schurink and R. Luttge, J. Vac. Sci. Technol., B 31, 06F903 (2013)]. However, trapping the neurons reproducibly under gentle, biocompatible conditions remains a challenge. The current setup involves the use of a hand-operated syringe that is connected to the back of the μSEA chip with a polydimethylsiloxane (PDMS) construct. This makes the capture process rather uncontrolled, which can lead to either cell damage by shear stress or the release of trapped neurons when unplugging the syringe and PDMS constructs. Although, the authors could achieve an efficient capture rate of single neurons within the 3D micropores (80%–90% filling efficiency), cell culture performance varied significantly. In this paper, the authors introduce a passive pumping mechanism for the parallel trapping of neurons onto the μSEA chip with the goal to improve its biological performance. This method uses the capillary pumping between two droplets (a “pumping droplet” on one side of the chip and a “reservoir droplet” on the other side) to create a stable and controllable flow. Due to simplification of the handling procedure, omitting the use of a syringe and additional connections to the μSEA chip, the set-up is compatible with real time microscopy techniques. Hence, the authors could use optical particle tracking to study the trapping process and record particle velocities by video imaging. Analyzing the particle velocities in the passive pumping regime, the authors can confirm a gentle uniform particle flow through the 3D micropores. The authors show that passive pumping particle velocity can be tightly controlled (from 5 to 7.5 to 10.4 μm/s) simply by changing the droplet volume of the pumping droplets from 20, 40, and 60 μl and keeping the reservoir drop constant (10 μl). The authors demonstrate that neuron capturing efficiency and reproducibility as well as neuronal network formation are greatly improved when using this passive pumping approach. © 2017 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/). https://doi.org/10.1116/1.4991827

I. INTRODUCTION

Microsystems for biology typically use microfabricated platforms (micrometer range) with MEMS and microfluidic components to study concepts ranging from electrical engineering to chemistry and biology.2 Lab-on-a-chip technologies, for which the idea is to miniaturize a fully operational laboratory on-a-chip of a few centimeters in size,3 have led to the development of organ-on-a-chip concepts where researchers are able to reconstruct part of human organs or recapitulate specific organ functions on-a-chip.4 Some examples include liver,5 kidneys,6 and lungs7 on-a-chip. In this research, our end goal is to create a platform technology for brain-on-a-chip8 to study and better understand epileptic seizures. This in vitro model can also potentially be extended to other brain disorders such as Alzheimer’s and Parkinson’s. The development of in vitro brainlike tissue constructs is also important for the understanding of healthy brain physiology. In vivo studies are slow, low throughput, complex, costly, and arguably immoral.9 In addition, animal use could be dramatically reduced for drug screening studies if relevant human in vitro organ-on-chip models were engineered, as they have high-throughput, reproducibility, and robustness, as well as cost-effectiveness considering today’s demand in pharmacological developments.9 Consequently, there is a need for improving current neuronal cell culture models to create in vivo-like brain tissue constructs on-a-chip. These miniaturized analytical displays can be used to study brain development and
complex brain cells’ interactions, leading to diseases in the form of novel biological assays. Engineering brain tissue constructs on-a-chip however is challenging and requires a multidisciplinary approach in the integration of a large variety of scientific skills. The brain is a complex yet highly organized network of cells communicating chemically and electrically with each other in a very specific manner and with an advanced hierarchical structure that enables its functionality. Neurological disorders and diseases arise when the brain cellular network is disturbed (i.e., by structural, biochemical, or electrical damage), which can lead to Alzheimer’s, Parkinson’s, and epilepsy, to name a few.

Growing neurons in a spatially standardized fashion (for example, in arrays) will ease the analysis of neurite connectivity (which is a hallmark neurodevelopmental end point indicator) and will lead to an easier method for relating changes in connectivity to electrophysiology and biological functions.

Our current concept on brain-on-a-chip has two main parts, a microbioreactor (MBR) enabling 3D cell/tissue cultures and a microsieve electrode array (μSEA) for pairing single neurons to electrodes (Fig. 1). The MBR serves as a 3D neuron culture chamber and contains a porous interface that will permit the diffusion of nutrients, and therefore, the neuronal cells are continuously fed via microfluidic principles. The μSEA enables the parallel trapping and pairing of neurons onto an electrode array containing 3D micropores and will be the main focus of this article. Commercially available microelectrode arrays (MEAs) already permit the recording and stimulation of neuronal activity but not to study events occurring for an individual neuron within a network over time. This is because cell cultures are dynamic and neurons migrate over time on a planar configured MEA, changing their locations on the array surface and making it impossible to follow the process over time. Accordingly, the MEA was modified into a μSEA to include 3D micropores at the electrodes in order to be able to trap hundreds of single neurons in a well-organized array conformation. The 3D micropores were also functionalized with an electrode matching the design of multichannel systems MEA readout electronics (Fig. 2). This enables an organized positioning of neurons and the formation of a spatially controlled neuronal network inbetween the 3D micropores. In this contribution, we demonstrate a new cell trapping procedure to capture single neurons within the μSEA without the need to use syringes or pumps but by exploiting passive pumping and capillary phenomena. This allows the generation of reproducible flow rates that are compatible with cell capture and cell survival whilst being also compatible with microscopy and eliminating the need for additional equipment.

II. EXPERIMENT
A. Microsieve electrode arrays

A μSEA chip is developed enabling hydrodynamic trapping of single neurons within highly uniform 3D micropores (Fig. 2). This μSEA chip has a surface area of several square millimeters. The 3D micropores are fabricated by means of corner-lithography and wet chemical etching in {100}-silicon. In brief, the fabrication process consists of a silicon sieving structure obtained by corner lithography with a patterned boron doped poly-silicon, connecting the contact electrodes within the 3D micropores. A LPCVD silicon-rich silicon nitride layer was used as insulation, and this new technology platform for multisite electrophysiology recordings was termed μSEA. A more detailed fabrication protocol can be found in the study by Schurink et al.

![Fig. 1. (Color online) Schematic representation of the brain-on-a-chip concept. A modified μSEA with single neurons paired to single electrodes is depicted also showing the 3D brain tissue construct cultured atop the μSEA within a so-called bioreactor.](image1)

![Fig. 2. μSEA (Ref. 13). (a) Brightfield image of the patterned and boron doped poly-silicon. The poly-silicon pattern forms the electrode layer consisting of contact electrodes and lead wires (scale bar 400 μm). (b) A cross-section of the circular area containing the 3D micropores with a thickness of approximately 16 μm, top micropores opening of 20 μm, and bottom apertures of 3.2 μm (scale bar 10 μm).](image2)
B. Cell culture and particle preparation

Polystyrene microparticles of 1 μm in size (Micromer®, micromod Partikeltechnologie GmbH) were diluted in phosphate buffer saline (PBS) to reach a final concentration of 500,000 particles/ml and were used to characterize the passive pumping flows. The neuroblastoma cell line SH-SY5Y (ATCC® CRL-2266™) was used to characterize the cell trapping procedures. The original cell line was isolated from bone marrow taken from a young human female with neuroblastoma.15 SH-SY5Y neuroblastomas were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM/F-12 media (1:1) supplemented with 10% fetal bovine serum and 1% pen/strep and grown in an incubator at 37°C, 5% CO2. When cell confluency was reached, trypsin (×1) was used to harvest the cells and centrifuged at 900 rpm for 5 min. A concentration of 200,000 cells/ml was used throughout the trapping experiments. Following the trapping, the cells were exposed to retinoic acid for 3 days at 10 μM in DMEM/F-12 media to differentiate the cells into neurons.

C. Trapping microparticles and SH-SY5Y neurons

Here, a passive pumping method was exploited to observe the capturing flow pattern of particles and consequently allow us to optimize capture conditions for trapping single neurons on the μSEA. First, a 10 μl empty (free of particles) droplet is placed under the μSEA (called reservoir drop, bottom side), and the chip is positioned into a clean Petri dish. A 20, 40, or 60 μl droplet (called pumping drop) containing the microparticles is immediately placed on top of the μSEA chip (top side), and the platform is positioned under the microscope for video recording. For the cell trapping procedure, the same approach was applied, but only 20 μl pumping drops were used.

D. Tracking microparticles

In order to characterize particles’ and cells’ flowing speeds and trapping velocity, particle tracking software (IMAGEJ, Mosaicsuite plugin) was used.16 Using these image-processing algorithms, videos of particles flowing inside the 3D micropores were analyzed for three different volumes of the pumping droplet (20, 40 and 60 μl). The particle tracking detection parameters had to be tuned for every video. Particle detection was carried out where the approximate radius of the particles was optimized (1 μm); the cut-off score for nonparticle discrimination was constant and the percentile which determines which bright pixels are considered as particles was the most variable. In addition, particle linking parameters such as displacement (maximum pixels a particle can travel between frames) and link range (to match the optimal correspondence matching) were also adapted for every video. To estimate the speed of the particles, first, the total pixel changes in both x and y axis were recorded. The total pixel changes were converted to micrometer. Then, the total distance traveled in both x and y directions was calculated, and the full trajectory over time was deduced by calculating the hypotenuse value \( (x^2 + y^2) \) = square root of the distance traveled. Then, the total amount of frames was converted to seconds, and the total distance traveled by a particle was converted to micrometer per second.

III. RESULTS AND DISCUSSION

A. Modified microsieve electrode array (μSEA) setup

The μSEA chip was produced as previously described by Schurink et al. (Fig. 2).1,13,14 The original protocol for trapping single neurons inside the 3D micropores involves the use of syringes and pumps and some additional polydimethylsiloxane (PDMS) parts to provide an active pumping mechanism. The original setup13 and protocol are shown in Fig. 3. However, we noticed that following the trapping of the neurons, the capture efficiency was not reproducible (ranging from 21% to 90%)13 and most of the neurons did not survive the trapping procedure and therefore were not able to network inbetween the 3D micropores. There were several reasons for the limited applicability of the procedure in the original test setup. One is that the trapping cannot be visualized under the microscope due to the syringe/PDMS construct blocking the light path. So, there is no mean to check if the neurons are being trapped during the procedure properly. Second, the trapping of neurons needs to be performed in sterile conditions to avoid culture infections, and so, the pump and the syringe have to be used under a cell culture hood, which is cumbersome and impractical for the end user. Third, the removal of the bottom PDMS construct connecting the μSEA to the syringe can cause negative back-flow. This means that neurons flow out of the 3D micropores. Fourth, the capture flow speed cannot be tightly controlled as tubing and plastic syringes have dead volumes which can impact the reproducibility.
of the trapping procedure and requires time to adjust for every experiment. To summarize these points, a more user friendly and more reproducible trapping procedure was developed that is easy to be adopted by a state-of-the-art biologists’ laboratory. The new trapping protocol only requires a standard micropipette for controlled dispensing of the reservoir and pumping drops in the passive pumping to start the seeding procedure. The details of the process are described in Secs. III B and III C below.

B. Microparticle trapping and tracking by passive pumping

To improve the performance of the single cell trapping protocol, a passive pumping principle was investigated [Fig. 4(a)]. Passive pumping is a simple method for pumping fluids in a semiautonomous way,17 which eliminates the need for expensive or cumbersome external equipment. Passive pumping is a principle that relies on surface tension and adhesion forces present in a small drop of liquid to create a flow through the microchannel. In our experiments, however, the reservoir drop (10 \( \mu \)l) is flattened between the \( \mu \)SEA and a Petri dish, and the pumping drop (20, 40, or 60 \( \mu \)l) is placed on top [Fig. 4(b)]. When this occurs, there is a weak capillary driven force that is generated between the \( \mu \)SEA and the petri dish. This directs the flow from the pumping drop to the reservoir drop and consequently through the 3D micropores. For flow speed characterization experiments, the pumping drop consists of particles in PBS with a concentration of 500 000 particles/ml and the

![Diagram](image1.png)

Fig. 4. (Color online) Novel single cell trapping principle by passive pumping. (a) Passive pumping (Ref. 17) is a phenomenon that allows fluids to be pumped spontaneously through microchannels and can be used to trap single cells on the \( \mu \)SEA. (b) Pumping and reservoir drops are positioned on the \( \mu \)SEA, and the chip is placed inside a Petri dish. The reservoir drop flattens between the \( \mu \)SEA and the Petri dish, creating a weak capillary force that pulls liquid (indicated by the arrows) from the pumping drop and resulting in passive pumping through the 3D micropores.

![Diagram](image2.png)

Fig. 5. (Color online) Tracking of microparticles by passive pumping using IMAGEJ MosaicSuite particle tracker plugin. (a) Polystyrene microparticles (diameter 1 \( \mu \)m) were added to the pumping drop at a concentration of 500 000 particles/ml, and video footage was recorded with varying pumping drop volumes (20, 40 and 60 \( \mu \)l). (b) Analysis of microparticle tracking shows that increasing the pumping drop volume leads to a gradually higher flow rate, from 8.6 \( \mu \)m/s (20 \( \mu \)l pumping volume) to 13.3 \( \mu \)m (60 \( \mu \)l pumping volume).

The reservoir drop consists of PBS only. The flow rate is determined from the volume of the pumping drop with respect to the reservoir drop. We report that using this approach, passive flows are generated reproducibly. Next, we changed the pumping drop sizes from 20, 40 to 60 l to investigate the effect of the drop size on flow rates. Using IMAGEJ MosaicSuite particle tracking package, we analyzed the videos [Fig. 5(a)] for each droplet size and plotted the particle speeds as a measure of flow rates generated [Fig. 5(b)]. We demonstrate that not only are the flow rates extremely gentle, from 8.6 to 13.3 l/m/s, but they also appear to be dependent on pumping drop sizes. The average total pumping flow rates were determined to be between 2 and 2.5 l/min. Although there is a large spread in the data, the average seems to reveal a trend in that the bigger the pumping droplet, the faster the flow rates.

C. Neuron cell pairing by passive pumping

Following the successful loading and characterization of the microparticle flow rates, we chose a droplet regime of 10 l for the reservoir drop and 20 l for the pumping drop as an optimum for neuron trapping. Using the same passive pumping approach and using the capillary effect inbetween the μSEA and the Petri dish, we report that single neurons can indeed be trapped in the 3D micropores and paired to the integrated electrodes using this approach [Fig. 6(a)]. We also observe that cell survival (90%) and therefore the onset of the neuronal network [Fig. 6(b)] are not issues anymore using this approach compared with the active pumping approach, indicating that the neurons are able to survive this gentle trapping procedure into the 3D micropores of the μSEA chip. This observation is also supported by the low cell capture flow rates which are compatible with cell survival. Furthermore, the flow rates are noticeably lower when using cells instead of microparticles (5.81 l/m/s compared to 8.6 l/m/s). We hypothesize that the reason for the lower trapping flow rates is due to 2 factors: first, a cell is more buoyant than a polystyrene particle, which means that it will move slower in liquid. Second, a cell is considerably larger than a particle (10 μm vs 1 μm) and therefore experiences more drag force. The observed lower cell capture speeds by passive pumping are an actual advantage considering that shear forces through the 3D micropores were previously impacting cell survival while using the syringes and pumps. In addition, this passive pumping procedure allows the user to reproducibly perform the trapping within minutes, in a sterile setting without the need for extra pumping equipment or loading parts whilst being compatible with microscopy.

IV. SUMMARY AND CONCLUSIONS

Passive pumping offers a fast (minutes) and simple route for the spontaneous and gentle trapping of hundreds of single neurons in parallel within the 3D micropores of our modified μSEA, eliminating the need for pumping equipment. This will facilitate the acceptance of this technology by the biology community. The passive pumping principle can also be extended to other cell types for single cell analysis where there is a need to trap single cells in an arrayed format. Examples include cancer, stem cells, reproductive biology, and many more.

ACKNOWLEDGMENTS

This research was financially supported by the ERC Grant No. 280281 (MESOTAS) and the ERC-PoC MESOTAS SIEVE Grant No. 713732. The authors would also like to acknowledge Zishan Waheed for his contribution to the particle trapping experiments.

25 SIB Swiss Institute of Bioinformatics, Cellosaurus SH-SY5Y (CVCL 0019).