Application Note

CellASIC® ONIX live cell analysis platform for neural stem cell microenvironment control

Abstract

Neural stem cells (NSCs) are sensitive to microenvironmental cues, including cell-cell contact, cell-ECM interaction, nutrient and waste transport, as well as environmental oxygen composition. However, how these parameters in the microenvironment affect the stem cells' morphology, proliferation, and differentiation remains an open area for research. In this study, we demonstrated how the CellASIC® ONIX Microfluidic Platform with its microfluidic cell culture devices are capable of multi-parametric microenvironment control for NSC studies.

Introduction

NSCs are self-renewable and multipotent cells that are capable of generating various phenotypes of the nervous system[1]. First described in the subventricular zone of the adult mouse brain[2], NSCs have received considerable attention due to their potential for therapeutic use. Research efforts generally divide into three focus areas: expansion, differentiation and cell:cell interactions. While commercial sources for NSCs have grown rapidly, building a physiologically relevant *in vitro* NSC culture model to thoroughly study the biology behind NSC expansion, differentiation and cell:cell interactions remains challenging. In addition to two-dimensional (2D) culture flasks, researchers have attempted other culture technologies such as three-dimensional (3D) neurospheres[3], scaffolds[4], and microfluidics[5]. While each technology has separately shown that NSCs are sensitive to microenvironmental cues, there has been no unified platform enabling researchers to systematically control the microenvironment for NSC culture.

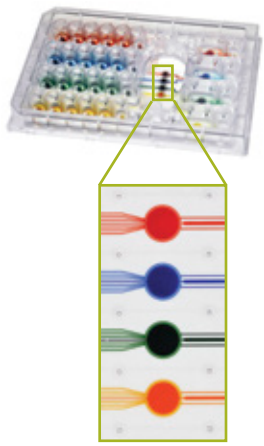

Figure 1.

(Above) CellASIC® ONIX Microfluidic Platform with the microfluidic system, the microincubator controller and microincubator manifold. (Right) Layout of M04S plate (four independent units and eight wells per unit).

The CellASIC® ONIX Microfluidic Platform offers comprehensive cellular microenvironmental control for cell culture studies (Figure 1). The ability to control multiple parameters has been integrated into the CellASIC® ONIX Microfluidic Platform to enhance the cellular microenvironment for NSC culture. To modulate the degree of cell-cell contact, the system can be set up to seed cells at varying density, leading to varying spatial distribution. To enable changes to cell-ECM interaction, we developed protocols to coat the #1.5 coverglass substrate with polyornithine/laminin, poly-D-lysine or poly-L-lysine. For nutrient and waste transport, both passive gravity-driven and pneumatically-driven control can provide low-shear, steady-state perfusion through NSC cultures with solution switching. Since the plate containing the cell culture chambers is made of gas-permeable materials, it is possible to inoculate NSC cell culture with different mixtures of gases. In this study, we show that the oxygen microenvironment can be tuned to range from severe anoxia to hyperoxia. To characterize NSCs grown in the microfluidic-controlled microenvironment, an automated immunostaining protocol was developed to visualize nestin and Sox2 within NSC cell culture.

We also tested the speed at which gas conditions could be changed using the microincubator, to assess the utility of the system for studying cell responses to hypoxia.

The successful combination of environment control with perfusion culture in a microfluidic platform promises to further close the gap between *in vitro* experiments and *in vivo* relevance.

Materials and Methods

Materials. Adult rat hippocampus neural stem cells and antibodies recognizing Nestin and Sox2 were obtained from EMD Millipore (Cat. Nos. SCR021 and SCR022). The CellASIC® ONIX Microfluidic System (Cat. No. EV262), Microincubator Controller (Cat. No. MIC230), Tri-Gas Mixer (Cat. No. GM230), and Microfluidic Plates (Cat. No. M04S-03-5PK) were also purchased from EMD Millipore. Poly-L-ornithine (Cat. No. P3655) and laminin (Cat. No. L2020) were acquired from Sigma. For poly-L-ornithine, the stock solution was prepared with sterile, deionized water to a final concentration of 50 µg/mL. Laminin was prepared with 1x phosphate-buffered saline (PBS) at 7 µg/mL.
The coating solutions were stored at 4 °C. All other reagents, such as rat neural stem cell culture medium (Cat. No. SCM009) and basic fibroblast growth factor (Cat. No. GF003) were obtained from EMD Millipore. For fluorescent microscopy, an Olympus IX-71 inverted fluorescence microscope with an automated stage was used. To validate the oxygen concentration in the cell culture chamber, an oxygen sensor from Presense was attached inside the microfluidic device.

Coating the cell culture chamber with extracellular matrices. Liquid was aspirated from wells 1, 6, 7, and 8 without aspirating liquid from the inner rings (see user guide for detailed protocol)[6]. 300 µL of poly-L-ornithine solution was then added to well 6. The plate was then placed inside a traditional cell culture incubator at 37 °C for 24 hours to coat the M04S microchamber. After 24 hours, the plates were retrieved and liquid in wells 6, 7 and 8 was aspirated. 300 µL of sterile, deionized water was used to rinse the well before another 300 µL of sterile water was added to well 6 to wash the microchamber. The plate was then placed in an incubator for 4 hours. For the second layer coating of laminin, the water in wells 6 and 7 was aspirated. 300 µL of the laminin solution was then added to well 6. The plate was returned to the incubator for 24 hours. Before seeding the cells, the laminin solution in wells 6 and 7 was aspirated. 300 µL of 1x PBS was added to well 1 and well 6. The device was then returned to the incubator for 2 hours to allow the PBS to gently wash out the remaining laminin solution in the microfluidic plate.

Cell seeding. The cell suspension was prepared according to the vendor's protocol and seeded at both high and low densities. For low density seeding, 1 x 10^5/mL was recommended. We seeded cells at high density (1 x 10^6/mL) to allow close cell-cell contact. Before introducing the cells into the microfluidic device, the liquid in well 1, 6 and 7 were first aspirated. The inner rings in well 6 and 7 were then carefully aspirated (extended aspiration will result in bubble introduction into the microfluidic channel). 10µL of the cell suspension (1,000 total cells for low cell density and 10,000 total cells for high cell density) was then added into the inner ring of well 6. The plate was then placed in a laminar cell culture hood for 30 minutes to allow the cells to load into the microchamber and settle.

Gravity-driven perfusion culture. To stabilize the cells, 300 µL of the culture medium was added to well 1 to allow gravity-driven perfusion and the plate was placed inside a regular incubator. For prolonged culture in the incubator, the medium in wells 1 and 7 was aspirated every 48 hours, and 300 µL of the culture medium was added into well 1 to re-establish gravity-driven perfusion. To culture the cells on the microscope, the PBS was aspirated from wells 2, 3, 4 and 5 but not the inner rings. As a guideline, 300 µL in each well, when flowed at 0.5 psi, could provide ample nutrients to the cell cultures for up to 36 hours.

Automated immunostaining. 300 µL PBS was added to well 1 as the wash solution, 100 µL of 4% paraformaldehyde was added to well 2 as a fixing agent, 100 µL of 0.2% BSA, 0.1% Triton®X-100 in 1x PBS was added to well 3 for permeation and blocking, 150 µL of primary antibody solution with 1% BSA was added to well 4, and 100 µL of secondary antibody was added to well 5. To conduct the immunostaining automatically, the pressure applied to each valve was programmed according to the following parameters: V2 (fixing), 4 psi, 12 minutes; V1 (washing), 0.25 psi, 8 minutes; V3 (permeation and blocking), 4 psi, 12 minutes; V1 (washing), 0.25 psi, 8 minutes; V4 (primary antibody), 4 psi, 60 minutes; V1 (washing), 0.25 psi, 8 minutes; V5 (secondary antibody), 4 psi, 40 minutes; V1 (washing), 0.25 psi, 5 minutes.

Testing NSC behavior with respect to gaseous microenvironment. First, we determined the gaseous microenvironment exchange rate. We purged the device in pure nitrogen and let it stabilize for over 24 hours. A pre-calibrated 5% CO_2 gas was then introduced through the microincubator. As depicted in Figure 2, the gas exchanged thoroughly in about 15 minutes. To investigate the impact of oxygen microenvironment on NSC behavior, we seeded the cells under the normoxia (20% oxygen) condition and let them stabilize in the incubator for 24 hours. We then transferred the NSCs onto three different microincubators and tri-gas mixers tuned to three gas compositions buffered with 5% CO_2 and nitrogen: severe hypoxia (~0.1% O_2), mild hypoxia (~3% O_2) and normoxia (~20% O_2). Loading 300 µL of the culture medium in well 2, 3, 4 and 5, we were able to culture the rat NSCs for up to four days, uninterrupted, through pneumatic pumping of medium across the cell culture chamber. Bright field images were acquired every day with a subset of cells immunostained for nestin and Sox2.

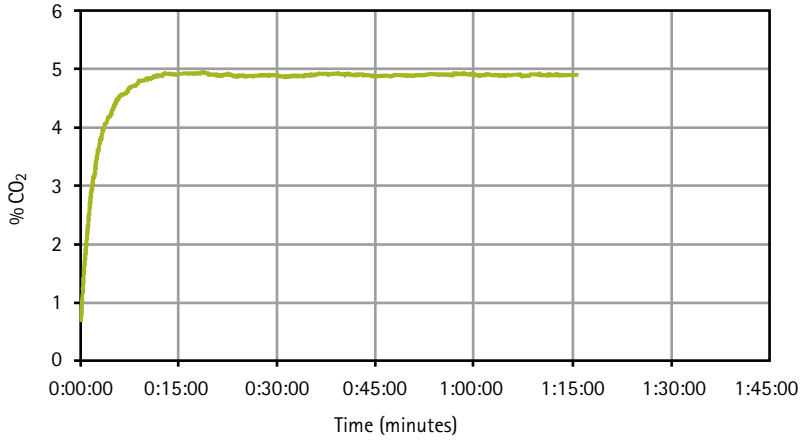

Figure 2.

The microincubator controller provided precise control of gaseous microenvironment for the microfluidic cell culture. Gas exchange was accomplished within 15 minutes.

Results

The key features of neural stem cells include self renewal and lack of differentiation. To examine these signatures in a gravity-driven perfusion microfluidic system over one week, we used automated immunostaining to detect the NSC markers, nestin and Sox2 (Figure 3). All the cells in the chamber were successfully stained, showing nestin in the cytosol and Sox2 in the nucleus, which confirmed that the rat NSCs maintained an undifferentiated phenotype while being cultured in this microfluidic device.

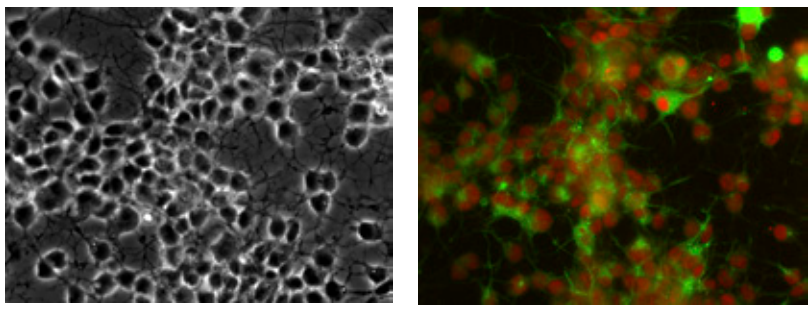

Figure 3.

Brightfield image of rat NSCs cultured in the CellASIC® ONIX M04S Microfluidic Plate on Day 8 (Left). Immunostaining of nestin (green) and Sox2 (red) indicated that these cells remained undifferentiated (Right).

We found that cells detached and washed away, presumably due to cell death, 24 hours after exposure to severe hypoxia (data not shown). At low cell density, cells disaggregated under mildly hypoxic conditions, which promoted single-layer cellular growth. In contrast, under normoxic conditions, the cells aggregated into multilayer cellular masses (Figure 4). Due to the cell loading variations, the initial seeding density (0 hour) in the normoxia chamber was higher than the mild hypoxia chamber (data not shown).

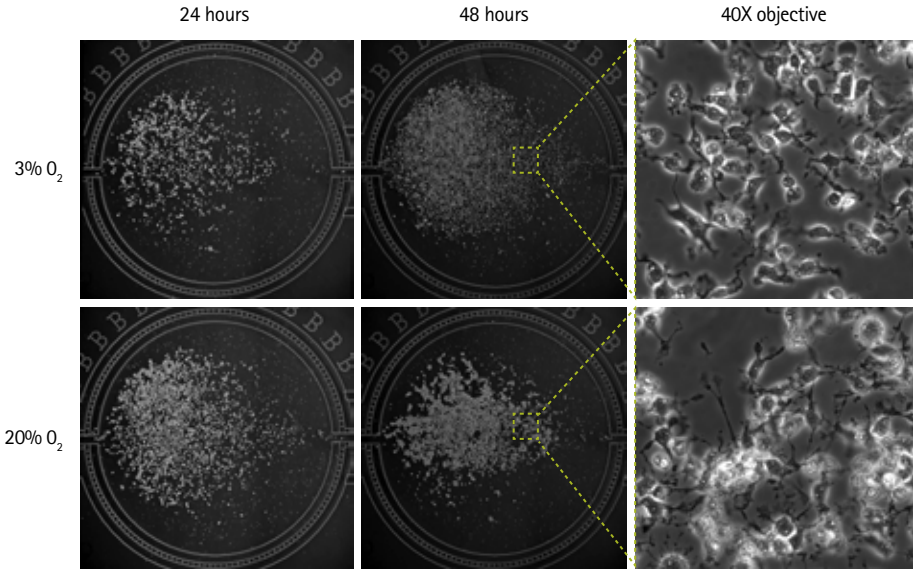

Figure 4.

Rat NSCs cultured at low densities under mildly hypoxic conditions (top) and under normoxic conditions (bottom). Under hypoxic conditions, the cells seemed to disaggregate and spread into discrete cell bodies while under normoxic conditions, the cells tended to aggregate.

To further explore the effect of microchamber culture of rat NSCs, we seeded the cells at high density under mildly hypoxic conditions (3% O_2). After four days, the cells formed neurospheres, though the spheres were compressed by being confined by the ceiling of the microchamber. Staining for nestin and Sox2 revealed that the core of this tightly packed mass contained only bright spots of Sox2 while the outer ring of the neurospheres exhibited both nestin and Sox2 expression (Figure 5).

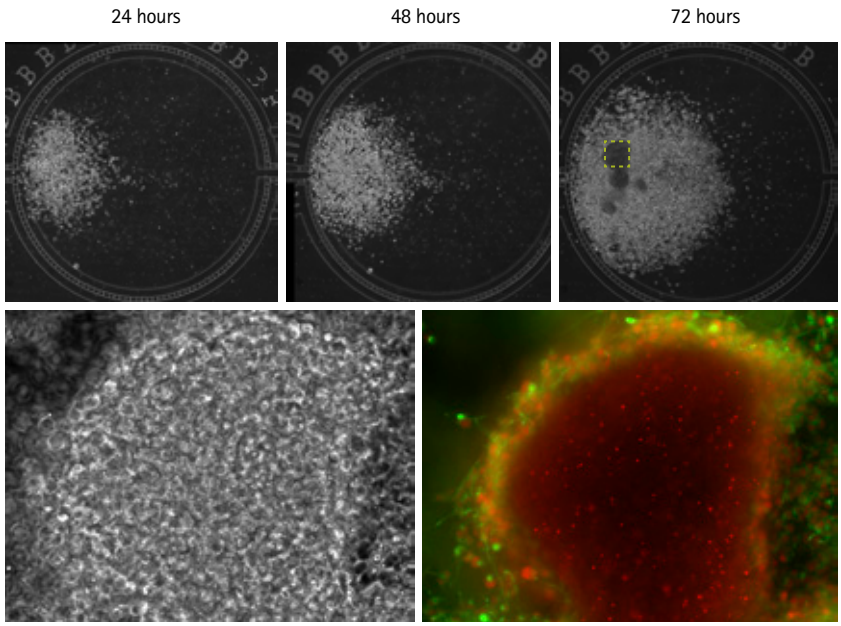

Figure 5.

High density seeding of NSCs in a microfluidic chamber under conditions of mild hypoxia causes formation of neurospheres (top row, inset box at 72 hours). Neurospheres were also visualized at higher magnification (20X, bottom left). When stained for nestin and Sox2 (bottom right), only the outer layers of the neurospheres showed both nestin (green) and Sox2 (red) expression; the core displayed bright spots of Sox2 expression. This pattern was possibly caused by neurosphere compression by the ceiling of the microfluidic chamber.

Discussion

The cellular microenvironment, and the oxygen microenvironment in particular, is known to regulate neural stem cell metabolism, proliferation, surivival and fate[7]. Mild hypoxia is a very physiologically relevant condition, given that the oxygen concentration in most tissues is lower than 20%, which is the atmospheric oxygen concentration ("normoxia"). Given that neural stem and progenitor cells have been reported to show increased proliferation and self-renewal under mild hypoxic conditions[8-10], studying the effect of NSCs with respect to gaseous microenvironment has the potential to advance research into the development of NSC-based therapies for neurodegeneration.

We have successfully developed the protocols to perform live cell analysis of rat NSCs using the CellASIC® ONIX Microfluidic Platform. By attempting various combinations of these microenvironment parameters, we found that the rat neural stem cells exhibited different morphologies and proliferated best under physiologic, mildly hypoxic conditions, as reported in the literature[8-10]. In addition, through the combination of high seeding density at 1 x 10^6/mL, polyornithine/laminin coating, continuous perfusion at 5 µL/hour, and 3% oxygen (mild hypoxia) gas microenvironment, the rat NSCs formed a 3 mm x 3 mm x 0.1 mm neurosphere in 96 hours.

We also investigated the effect of varying cell density on NSC differentiation. The importance of cell density on the differentiation of NSCs has been recently established by studies showing that NSC differentiation can be regulated by cell-cell signaling, possibly through the epidermal growth factor (EGF) and Notch signaling pathways[11,12]. Through live cell viewing on a fluorescent microscope, we discovered that, while the peripheral cells around the neurosphere were successfully immunostained for two pluripotency markers, nestin and Sox2, the image of the neurosphere itself showed condensed bright spots of Sox2 but no nestin, suggesting that increased cell-cell contacts within the neurosphere may affect NSC differentiation.

In summary, we have demonstrated the combinatorial effect of microenvironment parameters on rat NSCs and the capability of visualizing them using a standard fluorescent microscope for further analysis. The platform promises to facilitate assay development for NSCs and provides a better-controlled *in vitro* model system for neurogenesis and neural development research.

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