Application Note

Three-dimensional culture and assessment of drug-induced cell death using the CellASIC® ONIX Microfluidic Platform

Introduction

Cultured cells have proved to be convenient, powerful model systems for elucidating biological processes and for testing the safety, efficacy and mechanism of therapeutic candidates. The main objective in cell culture is eliciting cellular responses that match physiological responses as closely as possible.

In the context of tissues inside organisms, cells reside within three-dimensional (3D) extracellular matrices, frequently contacted on all sides by other cells and soluble factors. In contrast, traditional cell culture takes place typically on two-dimensional (2D) surfaces, on which cells are frequently flattened into non-physiological morphologies. In 2D cell culture, cells may not be able to access nutrients, intercellular signals or mechanical force feedback in the same way as in a 3D setting[1].

Numerous differences in responses between cells grown in 2D versus 3D culture have been reported. Hepatocytes have displayed higher cell viability and cell polarity in 3D spheroid culture compared to monolayer cultures, display more *in vivo*-like metabolic responses and also express more hepatic cell type-specific transcription factors[2,3]. 3D spheroids of endothelial cells (as well as mammary epithelial cells grown in an appropriate matrix) form hollow lumens in their centers, similar to *in vivo* vasculature[4,5].

Current strategies for 3D cell culture include growing cells in hanging drops, in a natural or synthetic 3D matrix, on biodegradable polymers, in a cross-linked hydrogel (such as Matrigel®) or in porous synthetic scaffolds consisting of interconnected voids in a woven mesh[1]. However, even these advanced cell culture platforms, if subjected to static conditions of gas, nutrient medium and waste buildup, are limited by the inefficient mass transport between the inside and outside of 3D cell structures[6].

To overcome the challenges of mass transport in 3D culture, the use of microfluidics (consisting of fluid flow paths on the order of microns in length and width) has gained in popularity. Microfluidics enables continuous flow from multiple angles in and out of a 3D culture, increasing the efficiency with which cells can access nutrients and eliminate waste[7]. This setup increases the longevity of 3D culture, enabling the study of cell responses over time, including responses with slow kinetics.

We used the CellASIC® ONIX Microfluidic System, in conjunction with the M04S microfluidic plate, to achieve perfusion-based microenvironment control and study drug-induced cell death of 3D cultures of MCF7 cells in Matrigel®. The microfluidic chamber recreates the physiologic mass transport condition for optimized cell health (Figure 1). Four upstream fluidic channels allow controlled exposure of the cells to different solutions. The plate can also be cultured in a standard incubator using a dedicated gravity-driven flow channel. The cells are in contact with a #1.5 thickness (170 µm) optical glass surface, enabling high quality viewing using an inverted microscope. An integrated micro-incubator system delivers temperature and gas control to the microfluidic chambers.

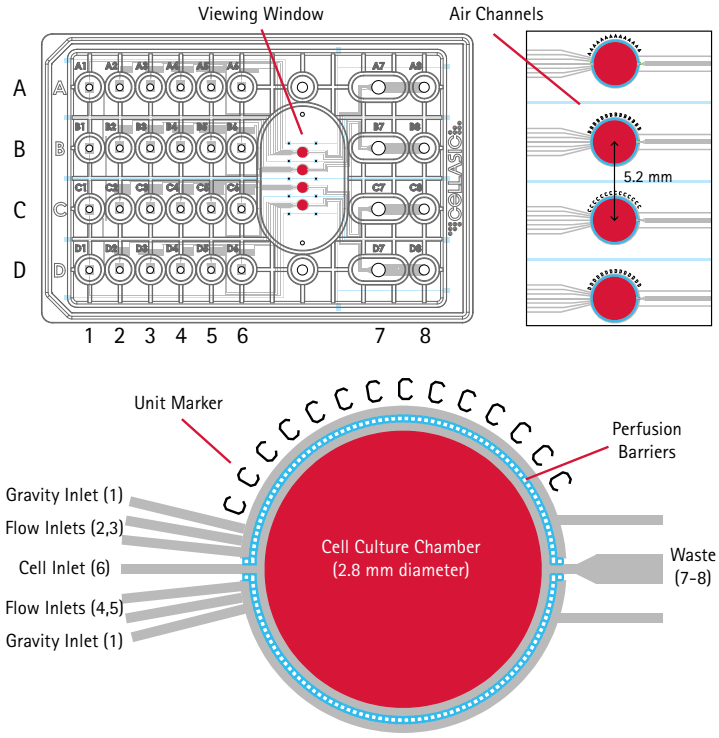

Figure 1.

The M04S plate contains four independent flow units (A–D), each with four upstream solution inlets, a gravity flow inlet, a cell inlet, and two waste wells. The culture chamber is 2.8 mm in diameter (120 µm height) and is surrounded with a microfabricated perfusion barrier. Inlet 1 is a gravity flow well, allowing cell culture in a standard incubator without a pressure system. Continuous flow of solutions from inlets 2-5 can create a dynamic exposure profile for viewing responses of live, 3D cultures.

Materials and Methods

We used the CellASIC® ONIX Microfluidic Platform for the 3D culture of MCF7 cells. The CellASIC® ONIX Microfluidic System (Catalogue No. EV262) delivered a controlled perfusion of the cells while the CellASIC® ONIX Microincubator Controller (Catalogue No. MIC230) provided the microenvironmental conditions required for healthy cell culture. The assembled CellASIC® ONIX M04S Microfluidic Plate and manifold with recirculating heater (Catalogue No. F84-HG3) were kept on the microscope and this allowed for live cell analysis of the cell culture.

Cell culture

MCF7 cells (ATCC®, Catalogue No. HTB-22) were cultured in Eagle's minimal essential medium (EMEM, ATCC®, Catalogue No. 30-2003) with 10% EmbryoMax® fetal bovine serum (FBS, Catalogue No. ES-009-B), 0.01 mg/mL bovine insulin (Sigma Catalogue No. I0516), and 1% penicillin-streptomycin (Catalogue No. TMS-AB2).

Initial cell counting and viability determination

For experiments, cells were trypsinized, harvested by centrifugation, and resuspended in complete medium. 50 µL of each cell sample was counted using the Scepter™ 2.0 handheld automated cell counter (Catalogue No. PHCC20060).

Culture of MCF7 cells in Millicell® EZ SLIDE

1.0×10^4 MCF7 cells in 100 µL of Matrigel® matrix were added to the wells of a Millicell® EZ SLIDE. Once Matrigel® matrix solidified, 400 µL of complete medium was added to each well. Cells were then incubated at 37 °C for 96 h.

Loading of cells into M04S plate

The M04S plate was placed in the refrigerator for one hour before loading, to ensure that the Matrigel®-containing cell suspension would not solidify during cell loading. All reagents were kept on ice prior to loading. Cells were suspended at 1.0×10^6 cells/mL in Matrigel® matrix by mixing a stock cell suspension with 10 mg/mL Matrigel® matrix at a ratio of 1:8. The temperature calibration plate was sealed to the CellASIC® ONIX manifold and calibrated to ensure that cell loading would take place at the desired temperature (equal to or lower than room temperature (RT)). Inlet wells 2-5 were aspirated and filled with 300 µL medium each. Well 6 was aspirated and then filled with 10 µL of the cell/Matrigel® suspension. The manifold gasket was wiped with 70% ethanol and the plate was then sealed to the manifold.

Automated protocol for cell loading, perfusion culture and doxorubicin treatment

Using the CellASIC® ONIX FG Software, an uninterrupted protocol was created in which inlet well 6 was set to

flow at 1 psi for 10 min (Step 1), for pressure-driven loading of cells. After cell loading was completed, the temperature was reset to 37 °C. To achieve continuous perfusion, each solution inlet well (wells 2-5) was filled with 300 µL medium, allowing scheduled medium flow from different solution inlets throughout the experiment. Flow rates for inlet wells 2-5 were set to 1 psi. After 48 h, the program was paused, plate unsealed and the waste well emptied. Plate was resealed and protocol resumed. After another 48 h, the program was paused again and the waste well was emptied. Wells 2 and 5 were replaced with medium containing different concentrations of doxorubicin (Catalogue No. 324380). The manifold was wiped with 70% ethanol, the plate was resealed and protocol resumed for another 48 h.

We changed media at 48 h due to experimental convenience, but longer intervals between changes may be acceptable. Because the automated perfusion program can be paused and resumed at any time, it is very simple to complete media changes during long-term culture.

Drug-induced cell death

The four chambers of the M04S plate were treated with four different concentrations of doxorubicin as follows:
Well A: no drug
Well B: 20 µM doxorubicin
Well C: 100 µM doxorubicin
Well D: 200 µM doxorubicin

Preparation for live/dead staining of MCF7 cells within microfluidic plate

The LIVE/DEAD® Viability Kit (Life Technologies) was prepared according to the manufacturer's protocol. In this assay, live cells, stained with Calcein-AM, fluoresce green, and dead cells, stained with EthD-1, fluoresce red. The M04S microfluidic plate was unsealed from the manifold and temperature set to RT. The wells to be used for live/dead staining were aspirated. 300 µL of live/dead stain was added to wells 3 and 4. 300 µL of phosphate-buffered saline (PBS, Catalogue No. BSS-1006-A) was added to wells 2 and 5. The manifold gasket was wiped with 70% ethanol. The plate was then sealed to the manifold and the CellASIC® ONIX FG Software was used to create and run the automated staining protocol.

Automated, in-plate viability staining

The M04S plate (as prepared above) was sealed to the manifold and placed on the lab bench and a two-step protocol was created in order to flow live/dead stain, followed by PBS, into the cell chambers:

1. **Step 1** (live/dead stain): Valves V3 and V4, pressure =0.5 psi, 60 min
2. **Step 2** (PBS wash): Valves V2 and V5, pressure = 0.5 psi, 5 min

Microscopy

All images were acquired with an Olympus® Q Color 5 Camera attached to a Zeiss Axiovert® inverted microscope, using ImagePro software. Scale bars are equal to 100 µm.

Results

Equal, even, pressure-driven loading of cell/Matrigel® suspension

Making biologically relevant conclusions by comparing one cell culture chamber to another in a single multi-chamber plate is only possible if cells are evenly loaded and subject to the same perfusion conditions in space and time. In the process of optimizing the cell loading protocol, we found that higher pressures and longer times were required for loading cells in Matrigel® suspension compared to loading cells in culture medium alone. (Typical pressures for loading cells in culture medium are in the 0.25 psi range, flowed over 0.1 min.)

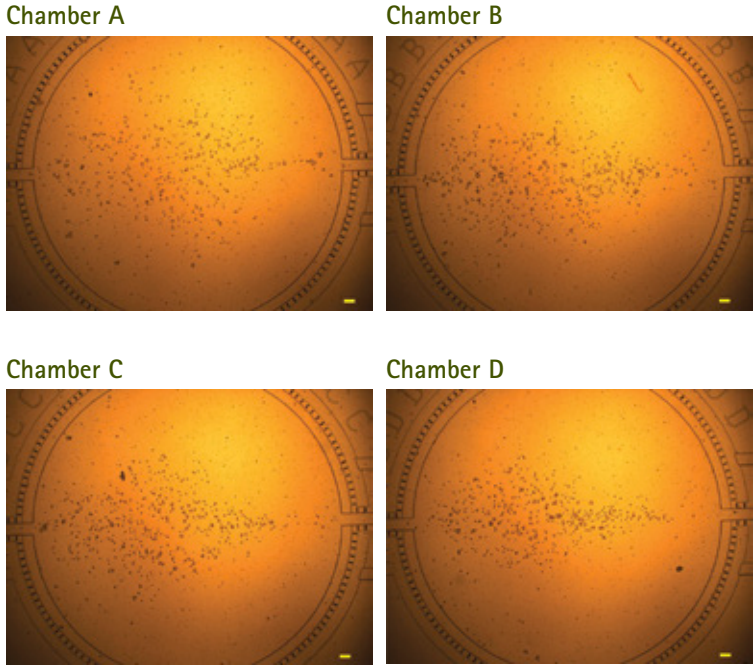

Figure 2.
Equal, pressure-driven loading of MCF7 cells in Matrigel® in four chambers of the M04S microfluidic plate.

Long-term perfusion 3D culture of MCF7 cells

As shown in Figure 3, after 24 h, MCF7 cells formed cell clusters that maintained their morphology after 96 h of culture in both perfusion culture (Figure 3A, 3C) and in static culture in the Millicell® EZ SLIDE (Figure 3B, 3D). Because growth of cell clusters was limited by the height of the microfluidic plate chambers, more cells were in sharp focus in the same focal plane than were in focus in the wells of the Millicell® EZ SLIDE.

Drug-induced cell death measured using the CellASIC® ONIX Microfluidic Platform

After 96 h of 3D culture, MCF7 cells were treated with doxorubicin for an additional 48 h. Cell viability was then assessed using in-plate live/dead staining. We observed the expected increase in red staining with respect to increasing doxorubicin dose, indicating that the 3D cell clusters were being permeated by the drug as well as by the live/dead staining reagents.

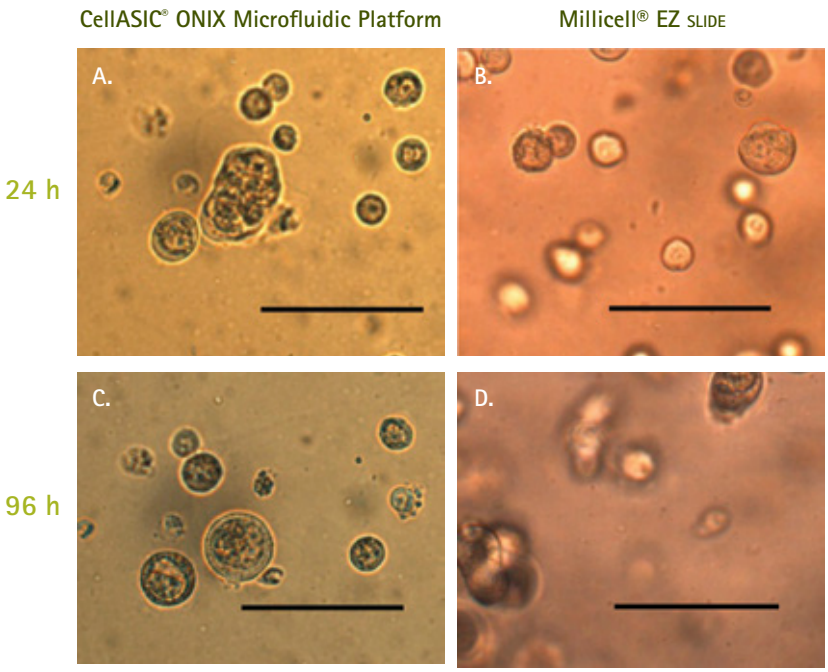

Figure 3.

3D cell cultures in sharp focus using the CellASIC® ONIX Microfluidic Platform (A,C). Images of cells were recorded at 24 h and 96 h. Static cultures conducted in parallel (B,D) showed similar morphology but fewer cell clusters per focal plane.

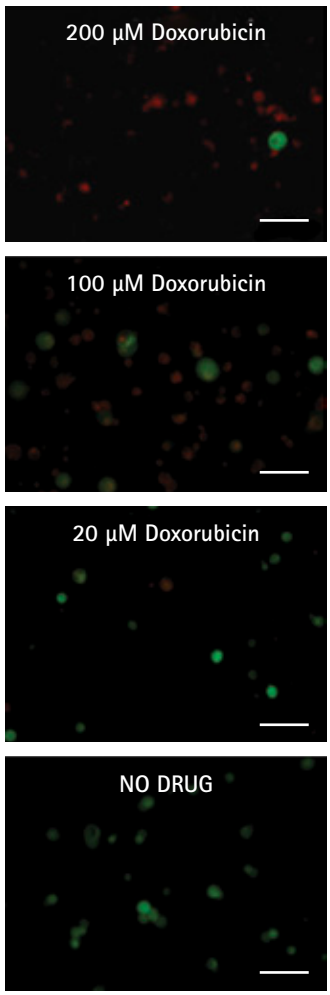

Figure 4.

Dose-dependent MCF7 cell death upon doxorubicin treatment as measured by in-plate live/dead staining of 3D cultures.

Discussion

We have shown that is possible to encapsulate cells in a hydrogel and set up evenly loaded 3D cultures in four chambers of a microfluidic plate using the CellASIC® ONIX Platform. As a result, we were able to simultaneously maintain four, virtually hands-free, healthy cell cultures for several days using perfusion feeding. 3D cell cultures in CellASIC® ONIX M04S Microfluidic Plates exhibited similar morphology and viability to parallel cultures kept under static conditions in a traditional incubator.

The cell loading conditions presented here are the optimized conditions for MCF7 cells in Matrigel® that we obtained by varying flow rates and durations. Given our observations, we recommend that users optimize these conditions for their own cell types and 3D culture matrices.

Further, we analyzed drug-induced cell death using hands-free, in-plate viability staining. This demonstrates that this platform enables the analysis of the response of individual cells within 3D culture. In addition, the CellASIC® ONIX Microfluidic Platform enables the culture of a larger number of cell clusters per focal plane, providing more precise data. The ability to analyze more clusters at once, evenly loaded in a multichamber plate, may provide increased statistical power to studies of cellular response and enable the routine use of 3D cultures for higher-throughput studies and screening.

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