Application Note

# Microfluidic perfusion enables long-term cell culture, precise microenvironment control and gene expression analysis

## Introduction

The analysis of living cells *in vitro* is critical to understanding basic biology, signaling pathways, drug effects, and disease models. Current methods provide excellent means to interrogate living cells via biomolecular probes, fluorescence microscopy, and genetic manipulation[1]. However, technology for environment control of living cells during analysis has not advanced significantly since the Petri dish. There is a growing body of evidence to indicate that the cellular environment, or "niche," is just as important (or even more critical) than genetic factors for determining cell phenotype[2]. Therefore, a method for providing more accurate, dynamic control of living cells has the potential to dramatically advance the state-of-the-art for live cell analysis[3,4].

The CellASIC® ONIX Microfluidic System, in conjunction with the CellASIC® ONIX Microfluidic Plate, provides perfusion-based microenvironment control for long-term, high quality, live cell analysis (Figure 1). The microfluidic chamber recreates the physiologic mass transport condition for optimized cell health. Four upstream fluidic channels allow controlled exposure of the cells to different solutions during live analysis. 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 microincubator system delivers temperature and gas control to the microfluidic chambers.

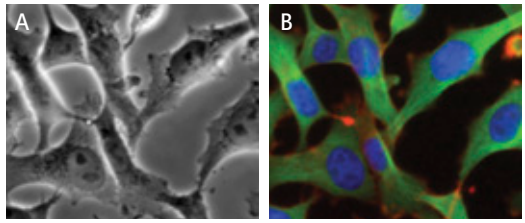



**Figure 1.**
Cells cultured in the M04S microfluidic chamber. (A) HT-1080 cells imaged under phase contrast and (B) immunostained for nuclei (blue), actin (red), and microtubules (green). Fixing and staining were performed within the microfluidic chamber. Images were acquired with a 40X objective lens.

In this study, we used the M04S Microfluidic Plate to demonstrate long-term culture of adherent cells, to create dynamic solution profiles (media switching and spatial gradient), to immunostain cells within the microfluidic chamber, and to analyze gene expression.

Because gene expression is influenced by numerous cell cultural parameters such as soluble biochemical factors, extracellular matrices, and stability of the microenvironment, accuracy and physiological relevance of gene expression analysis can be enhanced by performing such analyses in a dynamically controlled, bio-inspired, microfluidic system. Therefore, we demonstrate here the ability to conduct gene expression analysis using the CellASIC® ONIX Microfluidic System with M04S Microfluidic Plates. Ultimately, directly correlating gene expression patterns to phenotypes observed during live cell imaging can provide powerful, meaningful functional genomics data, revealing signaling networks and novel biomolecular interactions. Using breast cancer cell line MCF10A, we quantified the expression of Epidermal Growth Factor Receptor (EGFR) using standard quantitative reverse transcription polymerase chain reaction (qRT-PCR) techniques.

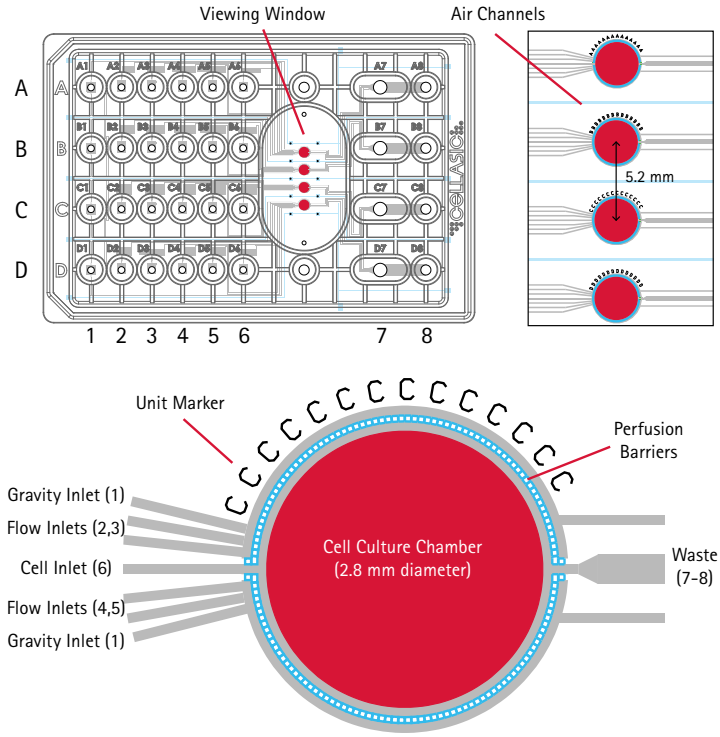



**Figure 2.**

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 long term cell culture in a standard incubator without a pressure system. Continuous flow of solutions from inlets 2-5 creates a dynamic exposure profile during live cell analysis.

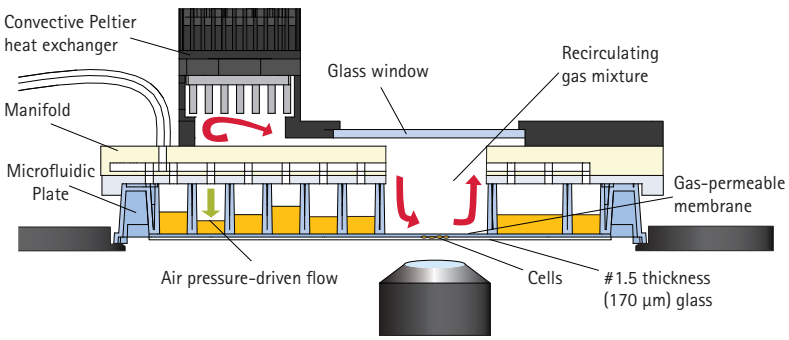



**Figure 3.**

Side view schematic of the microfluidic plate with micro-incubation manifold on a microscope stage. The bottom surface of the microfluidic plate is a thin glass sheet, allowing high quality cell viewing. The plate is sealed to a pneumatic manifold, allowing user control of the flow profile during analysis. Additional air channels allow control of the gas environment.

# Materials and Methods

## The CellASIC® ONIX Microfluidic System

The M04S Microfluidic Plate has a 96-well footprint to fit to typical microscope stage holders. The microfluidic chamber (four in each plate) has been designed to be compatible with standard culture methods, including surface coating, 3D culture, and co-culture. The bottom surface of the chamber is plasma-activated glass, which supports most standard cell lines without coating, including HeLa, NIH3T3, MCF7, MCF-10A, HT1080, HUVEC, and others.

The custom well layout maximizes live cell viewing capabilities. The M04S has four independent flow units (A-D), with each unit containing eight inlet and outlet wells (one gravity inlet, four switching inlets, one cell inlet, two waste outlets). The four cell culture chambers are centralized under a single large viewing window (Figure 2). The chamber-to-chamber distance is 5.2 mm, reducing objective travel time and focus drift. The bottom surface of the plate is a #1.5 thickness (170 µm) optical glass slide to maximize quality of high resolution, high numerical aperture viewing. The plate houses all experiment solutions, allowing control with an external pneumatic manifold (Figure 3). The manifold lets the user direct flow rates and select exposure solutions without perturbing the microscope stage. A gas line allows control of the environment within the microchambers through a network of gas-permeable air diffusion channels. Temperature is regulated through an on-board heater/chiller on the manifold.

## Cell loading

Cells are loaded into the culture chamber using a capillary-driven method. This allows the user to load cells using a pipette, and can be done in a sterile laminar flow hood without any external systems. The cell suspension is pipetted into the cell inlet well (6) and liquid is aspirated out of the waste wells (7 and 8). This creates a surface tension force that pulls the cell suspension into the chamber. As the cells enter the chamber, the flow profile allows them to settle to the floor without any stress. Typically, loading into the microchambers occurs within 3 minutes and cells are allowed to settle for up to 30 minutes. If more cells are desired, higher concentrations (or repeated loading cycles) can be implemented (Figure 4).

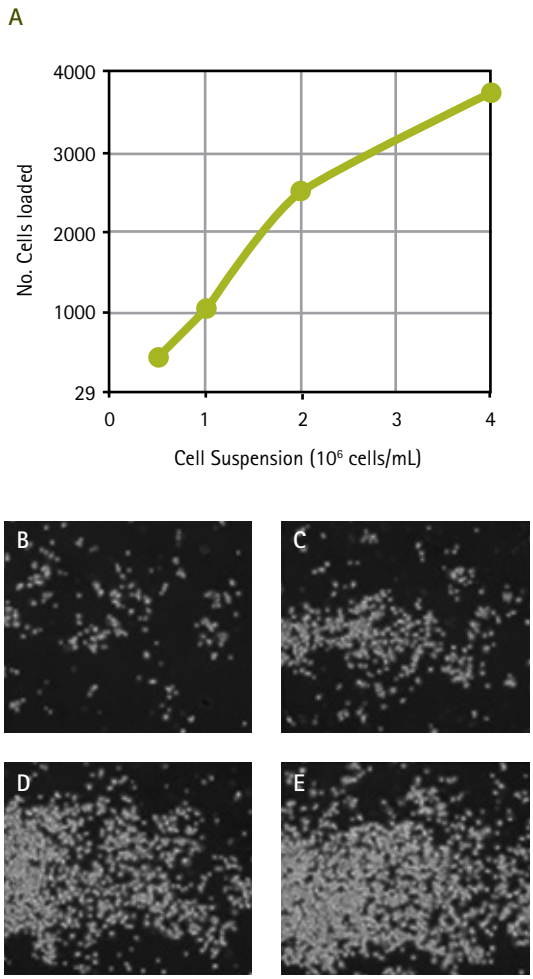



**Figure 4.**

Cell loading in the microfluidic chamber. (A) Each chamber can be seeded with up to a few thousand cells by varying the cell density during capillary flow loading. Images taken with a 4X objective of HT-1080 cells loaded at (B) 0.5, (C) 1.0, (D) 2.0, and (E) 4.0 million cells/mL.

After the cells settle to the bottom of the chamber, continuous flow of medium enables them to attach and grow. The chamber surface can be coated prior to cell loading by flowing the coating solution (e.g., poly-D-lysine, collagen, fibronectin) into the chamber using the same method. Alternatively, three-dimensional (3D) culture can be achieved by mixing the cell suspension with a gel matrix and loading together, allowing the cells/gel to polymerize within the chamber. The design of the flow channels allows continuous perfusion even in the presence of 3D gel.

## Gene expression analysis

Non-neoplastic human mammary MCF-10A epithelial cells (obtained from ATCC® (CRL-10317) ) were grown in Dulbecco's Modified Eagle Medium F-12 (DMEM/F-12) supplemented with 2% horse serum (Gibco), 5 ng/mL EGF (Invitrogen), 0.5 µg/mL hydrocortisone (Sigma), 100 ng/mL cholera toxin (Sigma) and 10 µg/mL insulin (Gibco) in a 5% $CO_2$ atmosphere at 37 °C. Prior to cell loading, MCF-10A cells were prepared in a cell suspension of 1 X $10^6$ cells/mL in the growth medium. Upon opening the M04S microfluidic plate packaging, priming solutions were aspirated from medium inlet well 1, cell inlet well 6, medium outlet well 7 and cell outlet well 8. After 10 µL of cell suspension was loaded into well 6, medium in well 7 was aspirated again to distribute cells evenly by capillary action. Plates were then laid flat in the incubator for an hour which allowed cells to settle without any flow disturbance. After one hour of incubation, 300 µL of growth medium was added to medium inlet well 1 and 50 µL of the culture medium into well 7 to initiate gravity driven perfusion of growth media during cell culture. Plates were then placed in the incubator until cells were confluent.

To lyse the cells, medium was aspirated from medium inlet well 1, medium outlet well 7 and 8, followed by pipetting 50 µL of 1X PBS (Cellgro) into well 1. After sealing the plate to the manifold, the CellASIC® ONIX FG Software was used to apply 4 psi on well 1 for 1 minute to rinse the channels and wash out cell debris in the chambers. The manifold was then unsealed and all liquid in well 1, 7, and 8 was aspirated. Then, 50 µL of the RNA extraction RNeasy™ RLT buffer (Qiagen) was added to well 1. After sealing the plate to the manifold, a cell lysing program was initiated on the CellASIC® ONIX FG Software (4 psi on well 1 and 0.25 psi on well 6 for 20 seconds). We then unsealed the manifold and collected 20 µL of the cell lysates from well 7. RNA was extracted using the RNeasy™ mini kit (Qiagen), following the RNA extraction procedures instructed in the manufacturer's manual.

Total RNA was then synthesized into cDNA using iScript™ cDNA synthesis kit (Bio-Rad) with random hexamer primers. Quantitative PCR was performed with the following primers: EGFR forward (5'- GGCAGGAGT-CATGGGAGAA-3') and reverse (5'-GCGATGGACGGGATCTTAG-3'). Samples were normalized using oligonucleotides specific to RNA encoding the house-keeping gene, GAPDH: forward (5'-ACCCACTCCTC-CACCTTTG-3') and reverse (5'-CTCTTGTGCTCTTGCTGGG-3'). cDNA was diluted 1:25 for GAPDH and 1:2 for EGFR before qPCR. Quantitative PCR was performed using SsoFast™ Evagreen® Supermix (Bio-Rad) on the MiniOpticon™ Real-Time PCR Detection System (Bio-Rad). The conditions for PCR were 95°C, 3 minutes followed by 40 cycles of 95°C 5 seconds, 60°C 10 seconds. Analysis variance and post hoc tests of quantitative PCR results were carried out using Bio-Rad MiniOpticon™ software.

# Results

## Solution switching

A key feature of the M04S plate design is that solutions can be changed during live cell analysis without perturbing the plate or microscope. This enables tracking of cell responses to changing solution environments. The M04S allows four different solutions to be switched during the course of the experiment.

Exposure solutions are introduced from the four inlet wells and flow through the chambers to the waste (well 7). Wells 7 and 8 are connected as a shared outlet for increased volume. Well 1 is a gravity flow well, with a perfusion rate of approximately 80 µL/day. This is used for pre-culture of cells in the M04S plate in a standard incubator when solution exchange or viewing is not necessary; for example, to expand or differentiate cells over a period of a few days. Wells 2-5 are the pressure-driven wells. The flow rate and exchange times are given in Figure 5. The highly laminar flow profile means that when the input solution is changed, a sharp fluid interface is created that moves across the culture area from left to right. The velocity of this front is given in Figure 5A. The actual local exchange time (the transition from solution 1 to 2 around the cells) happens quickly, typically in a few seconds. The small volume of the culture chamber enables fast solution exchange at flow rates from 5-80 µL/hour. This means that a typical experiment (with 300 µL per inlet well) can run for up to 72 hours.

As one example of flow switching, two solutions (phosphate-buffered saline (PBS) and dextran-conjugated fluorescein, 3 kDa, Invitrogen) were switched at 10-minute intervals (Figure 5B). Note the rapid and complete response of the solution, creating a clean "step function" in the culture region. Since all four channels converge near the culture chamber, the M04S plate minimizes the dead volume during switching to a few nanoliters.

In addition to flow switching, the CellASIC® ONIX Microfluidic System was used to create spatial gradients across a chamber (Figure 5C). When more than one channel was flowed simultaneously, laminar flow and diffusion across the interface created a stable spatial gradient. For sensitive kinetic experiments, it is recommended that a tracer dye be used to accurately follow solution flow profiles.

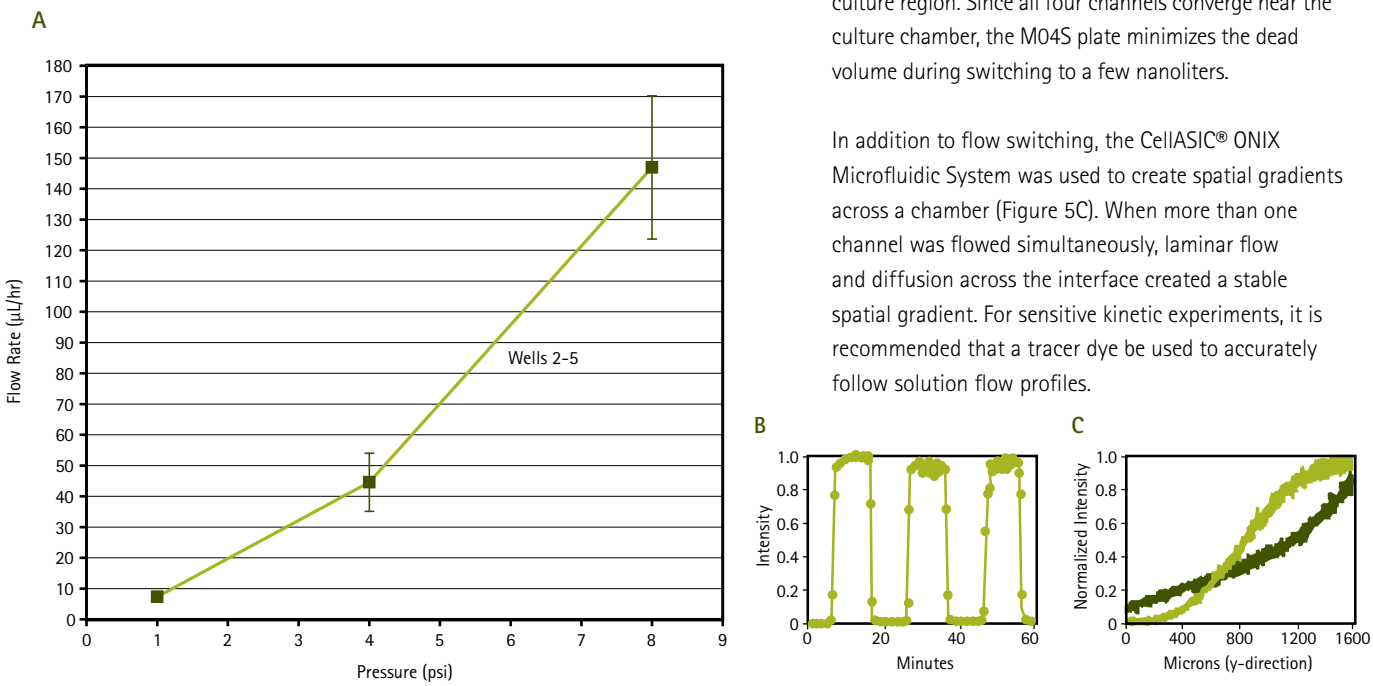



**Figure 5.**

A) Flow rate through the chamber as a function of pressure applied by the CellASIC® ONIX Microfluidic System to the inlet wells (V2 – V5) of M04S plate. The flow rates allow long-term, continuous flow experiments to be performed on the microscope stage. The inlet wells hold 350 µL of solution, allowing a single experiment to run for up to 72 hours. In general, a flow pressure of 1 psi is suitable for nutrient exchange. (B) Sequential switching between fluorescent dye and PBS. Intensity was measured in the center of the culture region every minute with flow at 2 psi from 2 channels. Data was plotted normalized to the max/min of intensity images. (C) Spatial gradient in the M04S chamber of fluorescein conjugated dextran (3kDa). Intensity was measured in the y-direction of the chamber. Light green line shows flow from 2 channels simultaneously of 0% and 100% dye at 0.5 psi each. Dark green line shows flow from 4 channels simultaneously at 0%, 25%, 50%, and 100% dye at 0.5 psi each. Diffusion across the interfaces creates a smoothed profile. Flow at faster rates will lead to sharper, step-like boundaries.

## Long-term cell culture

MDA-MB-231 cells were grown using the CellASIC® ONIX Microfluidic System in M04S perfusion plates for three days with continuous perfusion. Figure 6, showing the cells at four different time points, illustrates that cell health and morphology were preserved for the entire length of the experiment.

## Time lapse analysis

The favorable cell culture environment in the M04S chamber allows long term maintenance of adherent cells under well-controlled conditions. This enables enhanced live cell tracking of cellular events in response to changes in media, cell cycle, drug dosing, and other stimuli. We demonstrated two common methods for high resolution cell analysis: 1) immunostaining, and 2) transfection. To facilitate immunostaining of cells cultured in the M04S plate, an automated flow protocol was set up to sequence fixing, permeabilizing, blocking, primary antibody, secondary antibody, and all wash steps. This enabled monitoring of live cells in the M04S plate, and then subsequently fixing the same cells and analyzing by immunofluorescence.

In a second example, live cells cultured in the M04S plate were transfected with GFP-tubulin (Invitrogen Cellular Lights reagent). After exposure to the GFP reagent, the cells expressed the protein of interest and could continue to be tracked in the microfluidic system (Figure 7).

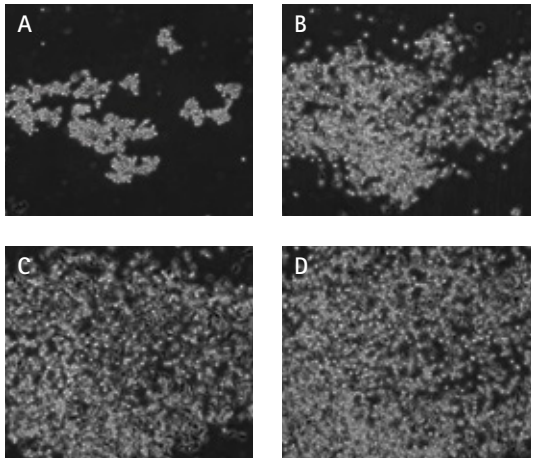



**Figure 6.**

Growth of MDA-MB-231 cells in the microfluidic chamber with continuous perfusion after (A) 1 hour, (B) 1 day, (C) 2 days, and (D) 3 days. Images were acquired with a 4X objective lens.

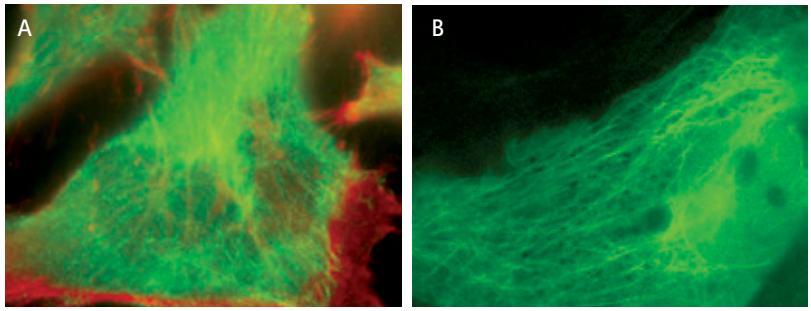



**Figure 7.**

On-chip staining of cells cultured in the M04S chamber. (A) HeLa cells cultured in the microfluidic chamber immunostained via flow exposure. Actin is shown in red, tubulin is shown in green. (B) MCF-10A cell cultured in the microfluidic chamber and transfected with GFP-tubulin via flow exposure. Images taken with a 100X objective lens.

## Gene expression analysis

We first quantified the total number of MCF-10A cells cultured to semi-confluency in each chamber of the four-chamber M04S plate using an Olympus microscope and ImageJ software (http://rsbweb.nih.gov/ij/). After counting the cells, we used an in-well lysis procedure and then removed the lysates for analysis. To demonstrate the efficiency of the lysis protocol, we grew cells to near confluency in the M04S Microfluidic Plate and obtained pictures of the cell culture chamber before and after lysis (Figure 8). The clear chamber after lysis indicated high collection efficiency, with no remaining cells left over in the chamber.

By conducting RT-PCR analysis of the collected lysates, we quantified EGFR and GAPDH gene expression in the cells. The EGFR expression per cell was normalized to GAPDH and total cell number in each chamber (Figure 9, top). The same experiment was performed on cells cultured on standard petri dishes. We found an almost 3X difference in per cell EGFR expression between the M04S plate and a standard petri dish (Figure 9, bottom). While we do not know the cause of this increase, it was highly reproducible and may reflect the environmental conditions supported in the microfluidic chambers.

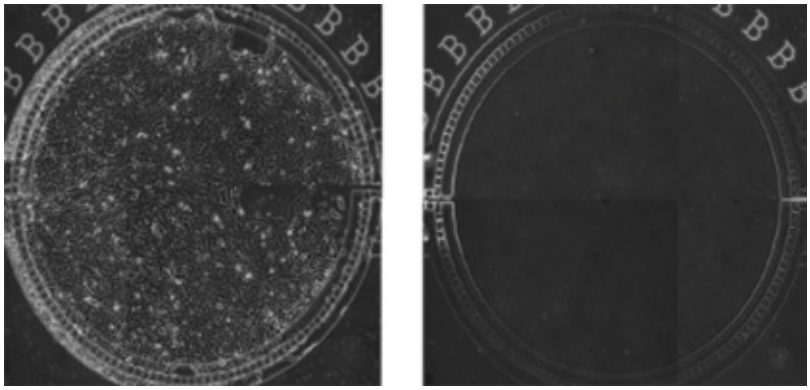
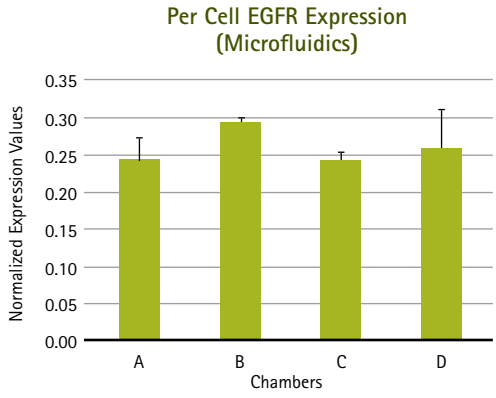


**Figure 8.**

Images of the cell culture chamber before (left) and after (right) cell lysis. The cells in the image at left were allowed to grow to confluency.



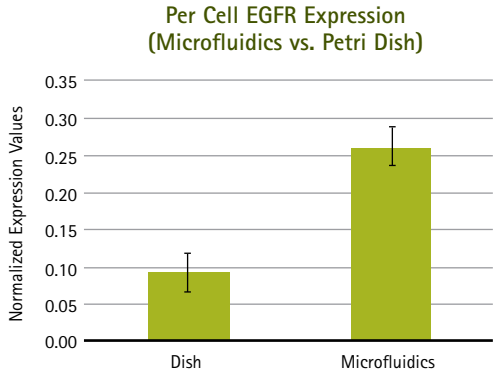




**Figure 9.**

(Top) Per-cell EGFR expression normalized to GAPDH expression and total number of cells per chamber. (Bottom) Comparison of per-cell EGFR expression of MCF-10A cells cultured in the CellASIC® ONIX System (M04S plate) vs. in standard Petri dish.

## Summary

The ability to control and monitor living cells is critical for understanding signaling networks and complex phenotypes in response to stimuli. We have developed the innovative CellASIC® ONIX Microfluidic System using the M04S perfusion chamber plate to optimize cell microenvironment control while facilitating long-term, high quality visualization. This design has been demonstrated with a wide range of cell lines for fluorescence quantification, solution exchange response, and time-varying inputs. Existing cell analysis methods such as immunostaining, transfection, fluorescent probes, and more, are easily adapted to the microfluidic format. Further, the ease of use, flexibility, and accessibility of this advanced technology platform should prove beneficial to a wide range of cell biology applications, including accurate, meaningful gene expression analysis.

## References

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