

## Application Note

# Microincubator for Long-Term, Live Cell Microscopy and Hypoxic Culture

### Introduction

Live cell imaging technology has made extraordinary progress in recent years, with improvements in microscopy, cellular probes, and genetic engineering<sup>1</sup>. The ability to perform live cell experiments within microfluidic chambers further extends the precision and biological relevance of *in vitro* studies<sup>2</sup>. One of the major technical challenges for long-term cell imaging is being able to control the temperature and gas composition of the cell environment during the course of the experiment, without impeding optical access to the cells. This is complicated by the proximity of microscope elements (objective lens, condenser, stage), as well as the need for an unobstructed light path through the sample without hindering stage or objective movement.

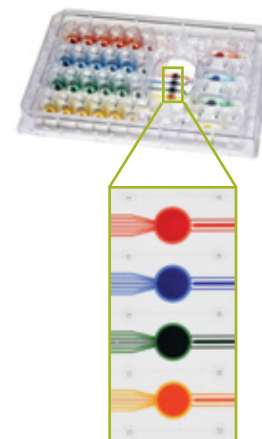
These challenges facing live cell analysis can be met by using microfluidics-based cell culture, which uses very small volumes of fluidics and culture chambers, to enable dynamic changes in gas and temperature conditions. Such a small thermal footprint allows the user to perform precise, quick changes of temperature or gas conditions during live cell experimentation, without interfering with optical analysis and enabling experiments that have traditionally been hard to perform. For example, the cost and inconvenience of performing experiments in static, hypoxic conditions have, until now, hindered the study of cell behavior in low-oxygen environments. However, having dynamic environmental control during live cell analysis can make such experiments possible. Therefore, a microfluidic platform is a promising basis on which to design an improved live cell incubation system with dynamic control.

Here, we demonstrate the application of the CellASIC™ ONIX Microincubator Controller (Figure 1) for long-term, live cell culture and imaging.



**Figure 1.**

CellASIC™ ONIX microfluidic cell culture system. The low-profile manifold (foreground) seals to the standard footprint microfluidic plate for imaging on any inverted microscope. Pressure-driven perfusion, temperature and gas lines route through the manifold to the microfluidic plate without blocking optical paths. The CellASIC™ ONIX Microfluidic Plate offers gaseous microenvironment control with rapid response through aeration channels and gas-permeable materials.



This system was engineered to create a "microincubator" space around the cell culture chambers in the microfluidic plate, allowing precise control of the temperature and gas environment with minimal footprint. Key advantages of this method include compatibility with inverted microscopes, a portable, self-contained cell culture system, minimal consumption of gas and heat, and rapid dynamics for temperature and gas change experiments. The CellASIC™ ONIX Microincubator was used with the CellASIC™ ONIX M04S mammalian culture plate to demonstrate long-term cell growth for over four days on the microscope stage.

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.

## Manifold design, materials and methods

### Temperature control

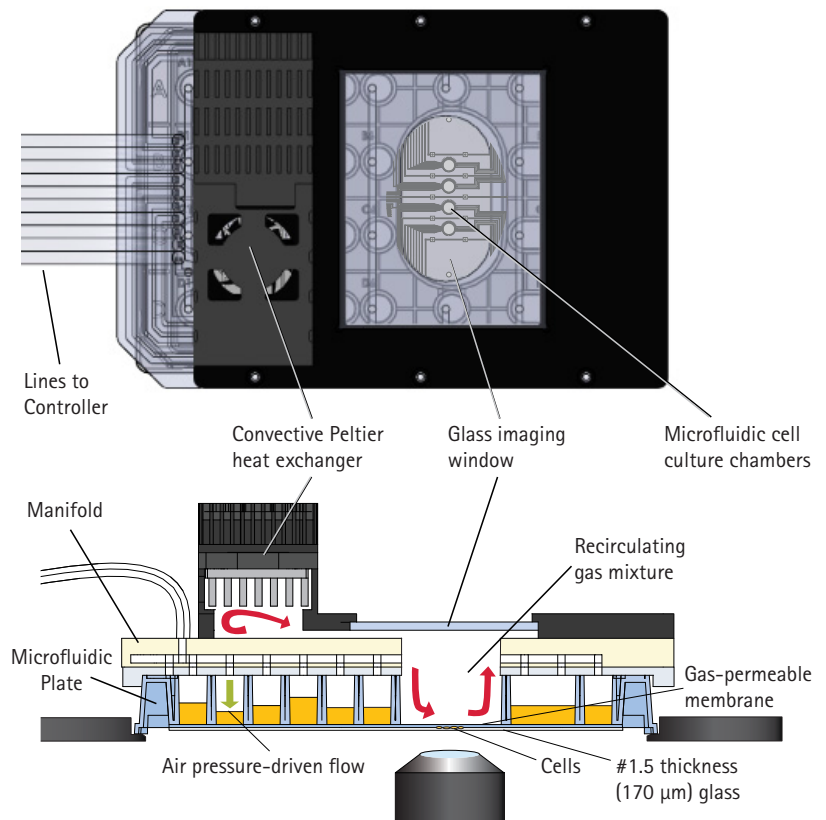
The CellASIC™ ONIX Microincubator utilizes an innovative recirculating heat exchanger manifold to regulate temperature and gas composition of the microfluidic

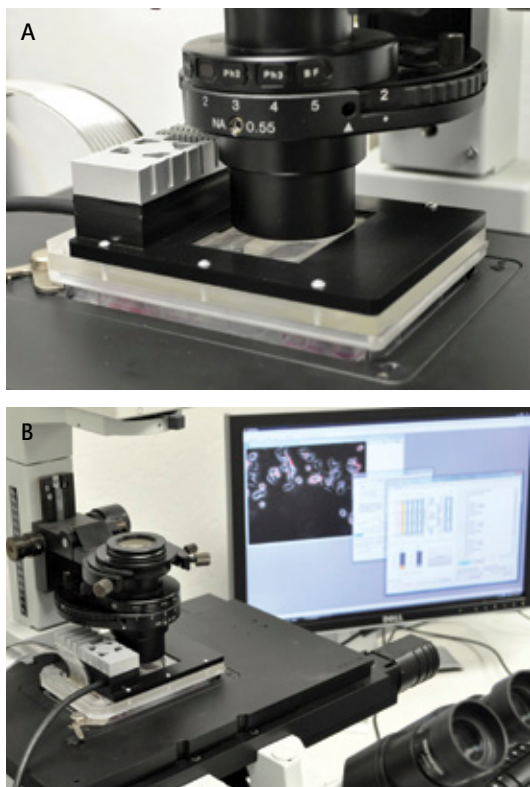
chambers. The manifold seals to the microfluidic plate, forming a 30 cm<sup>3</sup> enclosed incubation chamber (Figure 2). An on-board Peltier heat exchanger and circulation fans enable precise feedback control of the air temperature. The gas-permeable silicone barrier on the microfluidic plate enables the gas content of the microincubator to rapidly diffuse into the cell culture chamber. In most cases, the gas supply line is fed with 5% CO<sub>2</sub> for cell culture. However, this supply may be sourced from any premixed gas condition; for example 0.1% O<sub>2</sub> for hypoxia and 0% O<sub>2</sub> for anaerobic or nitrogen-rich environments.

The minimal thermal mass separating the microfluidic cell culture chamber from the heated air allows efficient heat transfer without requiring any heating elements on the bottom of the microfluidic plate. This ensures optimal optical quality when using an inverted microscope, giving the objective lens unrestricted access to the #1.5 thickness (170 μm) glass plate bottom (Figure 3). A window is cut out of the top of the manifold and covered with an optical glass plate for brightfield/phase/DIC illumination, preventing shadows and edge effects common to multiwell dishes. Since the manifold vacuum-seals to the top of the microfluidic plate without contacting the bottom surface, the assembly fits onto a standard 96-well plate stage holder, eliminating the need for microscope customization.

**Figure 2.**

CellASIC™ ONIX MIC manifold design. Top and side view schematics of the microincubator formed by the microfluidic plate and the heater manifold. The manifold seals to the top of the microfluidic plate, forming an enclosed volume for the recirculating gas mixture. The on-board heat exchanger regulates temperature. The manifold lines allow pressure driven flow control as well as delivery of premixed gas to the incubation chamber. Cells are cultured in the microfluidic chambers located under the imaging window. As shown in the side view, the M04S microfluidic plate contains four circular cell chambers, each with six inlet wells (left) and two outlet wells (right).





**Figure 3.**

Photographs of the manifold assembly on an inverted microscope stage. (A) The assembly fits to a standard 96- well stage insert, allowing direct access to the #1.5 thickness (170  $\mu\text{m}$ ) glass bottom for optimal image quality. (B) Software controls of the culture environment allow "hands-free" operation for over 96 hours during live cell imaging.

For accurate measurement of the chamber temperature, a microfluidic calibration plate was designed with an embedded thermistor probe. The probe was positioned in the center of the four cell culture chambers within the microfluidic layer. This was fabricated using a standard M04S microfluidic plate to give a direct reading of cell temperature, and to calibrate against external effects such as ambient temperature. When an immersion lens is used, the calibration plate allows accurate tuning of the objective heater to maintain desired cell temperature.

The performance of the CellASIC™ ONIX Microincubator was monitored under typical experiment conditions to measure stability, response to ambient temperature, and temperature shifts. For stability measurements, the chamber temperature was set to 37 °C. For temperature shift, a range of 30-37 °C was used. Measurements were logged using the calibration plate on an Olympus IX71 inverted microscope with continuous imaging of the chamber features using a 20X objective lens and flow of 5% CO<sub>2</sub> to simulate cell culture conditions. A second, identical probe was used to monitor ambient temperature outside the manifold.

### Gas control

Cylinders of highly accurate, pre-mixed calibration gas are readily available for nearly any desired gas environment. The CellASIC™ ONIX Microincubator has been designed to utilize these cylinders to provide specified gas conditions during cell culture. The manifold has a dedicated gas line (to which the gas cylinder is attached) that feeds the microincubator chamber. The desired premixed gas concentration is supplied to the microincubator. When the microincubator is on, it regulates gas flow to the proper flow rate to maintain a fixed gas concentration in the chamber without affecting temperature control. The gas fills the microincubation volume and exits through a vent in the side of the manifold.

This extremely low gas consumption enables one small gas cylinder to provide months of continuous culture. A Peltier heat exchange is integrated onto the microincubator manifold to provide temperature control and recirculate any desired gas mixture in the CellASIC™ ONIX microfluidic cell culture plates. The CellASIC™ ONIX microfluidic plates are constructed with gas-permeable materials and aeration channels, to readily allow pre-mixed gas to diffuse through the cell culture chambers and dissolve into the culture medium to provide cells with the desired gaseous microenvironment.

For live cell imaging, 5% CO<sub>2</sub> was used to support mammalian cell culture in standard medium. When used with a gas mixing or switching module, the microincubator allows timed delivery of dynamic gas concentrations to the cell culture chamber. The response time is approximately 10-30 minutes for the new gas to reach the cells.

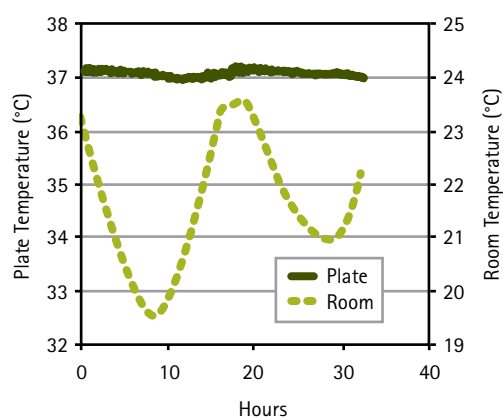
To demonstrate the gas control performance of the CellASIC™ ONIX Microincubator, we investigated the time required to switch between two different gas environments, hypoxic and normoxic. Using ruthenium tris(dipyridyl) dichloride hexahydrate (RTDP), a water-soluble oxygen-sensitive dye, we measured changes in oxygen concentration within the fluid in the microfluidic channels. Solutions of 1 mg/mL RTDP were loaded into CellASIC™ ONIX M04S plates and flowed at 2  $\mu\text{L}/\text{hour}$ . Fluorescence intensity measurements were obtained with an Olympus IX71 inverted microscope and 60X oil objective. To eliminate temperature variations as well as mimic typical experimental conditions, the temperature of the imaging region was maintained at 37 °C using the CellASIC™ ONIX Microincubator manifold F84-HG3 along with an objective heater.

## Results

### Temperature measurement

Temperature stability was found to be within 0.2 °C of the 37.0 °C setpoint, and was insensitive to fluctuations in the room temperature (Figure 4). In this experiment, the microincubator was monitored for 36 hours, over which the room temperature varied from 19–24 °C. The cell culture temperature during this time ranged from 36.9–37.2 °C. When plotted against each other, there was a weak positive correlation between the room temperature and the plate temperature ( $R_2 = 0.06$ ), with a slope of 0.01 °C change in plate temperature per degree change in room temperature. This indicates that under standard operating conditions, the microincubator is capable of maintaining a very stable cell culture temperature during live cell imaging.

A.



B.

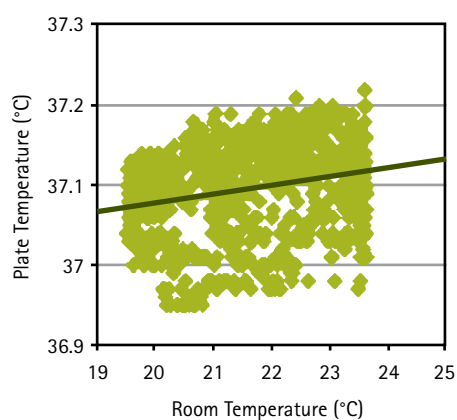
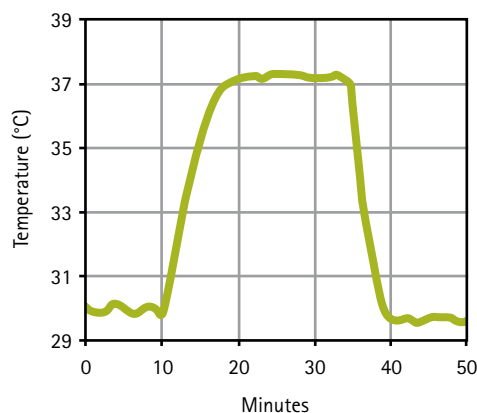


Figure 4.

Temperature stability of the CellASIC™ ONIX Microincubator during live-cell imaging. (A) Time course of microfluidic chamber temperature (“plate”) over 36 hours on the microscope stage, showing about 0.2 °C fluctuation. (B) Plate temperature plotted against room temperature.

The design of the CellASIC™ ONIX Microincubator makes it well suited for dynamic temperature changes, or temperature shift experiments. To test this performance, the chamber temperature was set from 30 °C to 37 °C and back (Figure 5). The heating time from 30–37 °C was 8 minutes, with a half-time of 3 minutes, and the cooling time from 37–30 °C was 7 minutes, with a half-time of 3 minutes. The rapid temperature change was possible due to the small thermal mass of the system. The room temperature was constant at 25 °C during the experiment.

A.



### B. Microincubator Information

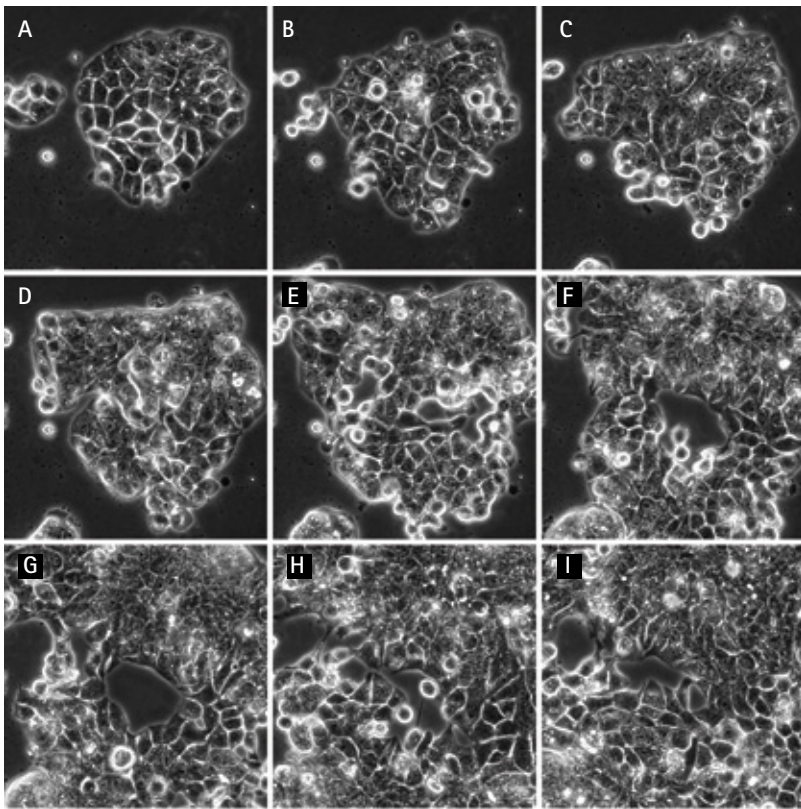
Temperature Range	RT – 40 °C
Achievable Temperature Stability	+/- 0.2 °C
Heat Time (30 – 37 °C)	8 min
Cool Time (37 – 30 °C)	7 min
Incubation Volume	30 cm <sup>3</sup>
Gas Flow Rate	3.5 mL/min +/- 0.5 mL/min
Condenser Working Distance (minimum)	26 mm
Imaging Window Size	25 x 42 mm

Figure 5.

(A) Temperature shift experiment ramping from 30–37 °C and down from 37–30 °C. The shift time was approximately 8 minutes. (B) Table summarizing microincubator information.

## Long-term cell culture

The CellASIC™ ONIX Microincubator was validated for long-term live cell microscopy using the M04S microfluidic plates. Here, a human colon adenocarcinoma cell line (HT-29, ATCC®) was cultured for 96 hours with continuous imaging on the microscope stage (Figure 6). The cells were perfused with culture medium (McCoy's 5A, ATCC®) at 5  $\mu\text{L}/\text{h}$  and an environment of 37.0 °C and 5%  $\text{CO}_2$  for the duration of the experiment. As evidenced by the images, the cells remained healthy and showed typical cell division rates and morphology. The system (including microscope and software) was completely unattended during the 96-hour run, demonstrating the "hands-free" operation capability of the microfluidic platform. Similar experiments were successfully repeated with a variety of adherent cell lines, indicating flexibility for multiple applications.

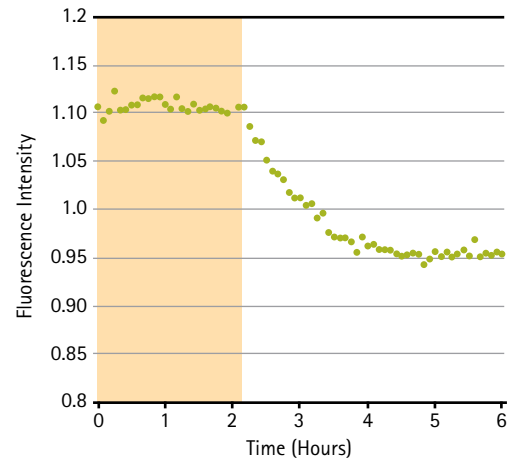


**Figure 6.**

Long-term HT-29 cell growth on the CellASIC™ ONIX Microincubator with continuous perfusion on the microscope stage at (A) 0 h, (B) 12 h, (C) 24 h, (D) 36 h, (E) 48 h, (F) 60 h, (G) 72 h, (H) 84 h, and (I) 96 h. Images were acquired with a 20X phase contrast objective lens.

## Hypoxic culture

Following a switch between hypoxic (100%  $\text{N}_2$ ) and normoxic (Air) conditions (2-hour mark in Figure 7) the fluorescence intensity dropped due to oxygen-quenching of the RTDP dye. The data indicated that the gaseous environment of the fluid in the cell culture region stabilized in about 2 hours following the switch (around 4 hour mark in Figure 7).



**Figure 7.**

Oxygen-sensitive dye RTDP indicated that the gaseous microenvironment was exchanged from hypoxic to normoxic conditions in around 2 hours.

## Conclusions

The CellASIC™ ONIX Microincubator is a breakthrough system enabling precise environment control for long-term microfluidic cell culture with clean integration with existing live cell imaging microscopes. In previous work, it was demonstrated that the CellASIC™ ONIX microfluidic culture chambers provided unique advantages for cell biology experiments<sup>4</sup>. However, the reliance on existing microscope temperature/environment control solutions limited accessibility of the microfluidic platform, while also proving inadequate for many applications. Here, we validated the performance of the CellASIC™ ONIX Microincubator to deliver precise and stable temperature and gas control for multi-day microscopy applications and for culturing cells under precisely controlled gas compositions. The combination of microfluidic perfusion culture, environment control, and optical quality makes the CellASIC™ ONIX Microincubator the ideal platform for long-term live cell imaging experiments that require precise gas environment control, such as hypoxia. The continued adoption of this technology will lead to further insights into physiologically predictive *in vitro* cell models and improved data relevance.

## References

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