Application Note

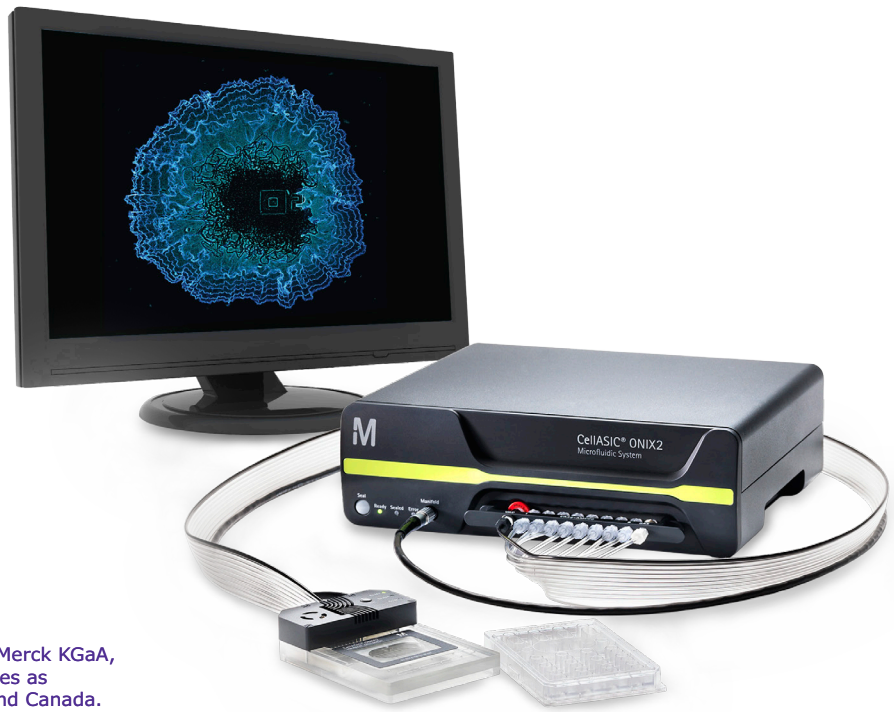
An integrated platform for real-time dynamic culturing and analysis of hypoxia with single cell resolution

Introduction

Hypoxia, or inadequate oxygen supply in tissues, is a hallmark of primary tumors, arising due to impaired vascularization and increased respiratory demand of rapidly proliferating cancer cells. Clinically, hypoxia-driven RNA signatures correlate with aggressive tumor behavior and poor disease prognosis, and hypoxia has a major impact on aspects of metastatic disease that include cell proliferation, metabolic capacity, immune response, and resistance to chemotherapeutic intervention. At the cell level, hypoxic responses are mediated by HIF (hypoxia-inducible factor) transcription factors which regulate the expression of genes that drive adaptation of resident cells. Unraveling the mechanisms underlying hypoxic responses is essential to developing therapeutics targeting tumor progression.

Standard cell culture has two major limitations for studying the effects of steady-state oxygen levels and hypoxia. First, media overlying cells presents substantial resistance to oxygen diffusion, resulting in a significant lag in cell-level gas changes relative to the gas phase. Second, and more significantly, due to convective mixing, diffusion resistance varies unpredictably, making precise control of oxygen pressure at the single cell level difficult to achieve. Moreover, many culture control devices are not expressly designed for microscope-based visualization, and therefore cannot be used for live imaging analysis of single cells. *In vitro* analysis approaches that have the capacity for precise regulation and maintenance of microenvironmental factors while permitting real-time imaging of tumor model development and response would confer significant advantages for oncology research.

In this application note, we study the hypoxic responses of various cancer cells using a combined cell culture/imaging platform for analyzing hypoxic responses in real time. Our data show that hypoxia impacts diverse parameters, including response to cytotoxic agents, mechanisms of cell death, and invasive capacity. Similar studies will help inform better strategies for preventing cancer progression.



Materials and Methods

CellASIC® ONIX2 Microfluidic System

The CellASIC® ONIX2 imaging platform combines media perfusion and micro-incubator (gas exchange and temperature) control in a single device, for automated long term cell culture with continuous live-cell imaging. The design permits exposure of cells to different environmental conditions via pressurized flow channels; culturing parameters are programmed through user-specific changes in media source and flow rate.

The culturing unit consists of a microfluidic plate and environmental control system. Micro-volume cultures allow more precise chemo-temporal and spatial control, including rapid changing of conditions. Plates are constructed with gas-permeable materials and aeration channels to minimize diffusive effects. The controller regulates pressurized cell loading, programmable media perfusion, temperature, and gas levels. Because of its compact size (96-well plate) and high optical clarity, the plate can be paired with most inverted microscopes for automated dynamic analysis. Fluorescent visualization further permits selective discrimination of unique cell types and changes in expression of labeled proteins.

Manifold:

The CellASIC® manifold unit interfaces with microfluidic plates for precise environmental control. The pneumatic manifold uses pressurized air to pump cells and liquids through microfluidic channels from source wells to the cell culture chambers. The vacuum seal with the microfluidic plate ensures each media reservoir is uniquely regulated for high accuracy in perfusion rates and source well switching. All culture parameters can be programmed and automated.

Plates used in this study:

The CellASIC® M04S (switching) long-term culture plate for mammalian cells is a four-chamber device with six independent inlet reservoir wells for cell loading and media addition and two waste outlet wells for continuous media cycling. The device is designed for long-term manipulative cell culture with real-time image analysis. The M04G (gradient) migration plate is a four-chamber device with three compartments per chamber. The upper/lower channels are for cell/media loading and the middle channel is for matrix loading. Gradients are established by simultaneously flowing media of different compositions through the upper and lower channels.

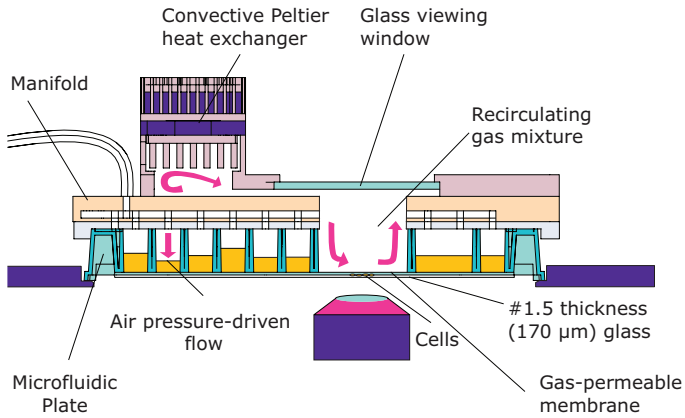
Cell Lines

Human cell lines (MDA-MB-231, M4A4, A431, A549, MCF-7, HT1080, HCT116, HeLa) were maintained under standard growth conditions. For migration/invasion studies, cells were starved for 24 hours prior to performing assays.

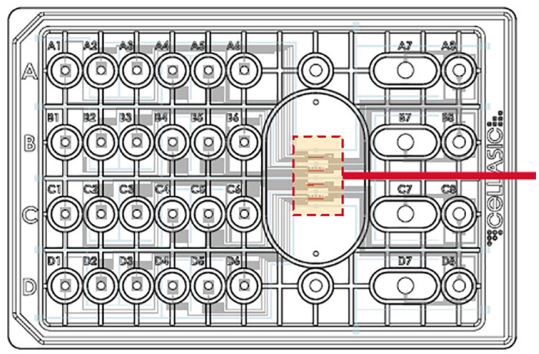
Image Capture and Analysis

Images were acquired using the Lionheart™ FX cell imager (BioTek) at 10X and 20X using bright field and fluorescent filters. Time-lapse imaging was performed at various time intervals during the studies. Static images of fluorescent intensity were analyzed using Gen5™ software; Image J software with Manual Tracking and Chemotaxis tools (NIH) was used to analyze invasion assay images.

ONIX2 controller



Manifold/Plate



Base Microfluidic Plate

Figure 1. Diagram of CellASIC® system components: the ONIX2 controller, manifold, and base microfluidic plate.

Results

Verification of gas control by the ONIX2

The ONIX2 controller has two independent gas inlets for automated switching of micro-environmental gas content. To demonstrate gas control performance, RTDP [ruthenium tris(2,2'-bipyridyl) dichloride hexahydrate], an oxygen-sensing fluorescent dye, was used to assess stability and switching capacity (**Figure 2**). On average, each switch took two hours to stabilize. The hypoxic state was maintained for 48 hours. Due to the nature of gas delivery in the ONIX2 system, media content, flow rate, and presence of cells had no impact on O_2 concentration.

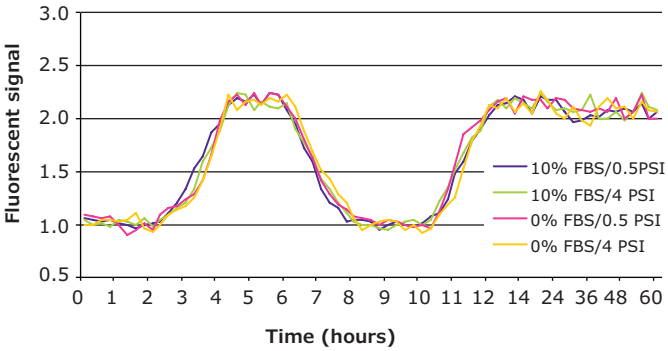


Figure 2. RTDP-containing media (1 mg/mL) was perfused at constant flow rate with fluorescent image collection at 10 minute intervals. Upon switching gas content (normoxic/hypoxic, arrows), fluorescent intensity shifted coincident with changes in oxygen quenching of RTDP.

Hypoxia induction alters cellular apoptotic potential—switching plate assays

Under normal circumstances, hypoxia is cytotoxic. However, tumor cells often acquire the ability to adapt and thrive in hypoxic conditions. Experimental and clinical evidence demonstrates a strong correlation between these adaptations and resistance to chemotherapy and radiation therapies, as well as the progression of malignancy. Recent findings have also suggested that oxygen treatment has the potential to reverse chemo-resistance.

We show that hypoxia pre-conditioned cells had a reduced response to cytotoxic agents staurosporine (STX) and camptothecin (CAM) (**Figure 3**). This effect was transient and could be reversed by re-exposure to oxygen. Response to TNF-related apoptosis-inducing ligand (TRAIL) was not affected by oxygen reduction; unlike chemo-agents, receptor-mediated apoptosis bypasses the mitochondria a primary target of hypoxia- inducible factors.

At various time points during the hypoxic conditioning and normoxic re-establishment, chambers were washed, fixed, permeabilized, and stained to identify total cells and the hypoxia-responsive fraction (**Figure 4**). Hif1α levels correlated with changes in chemo-resistance, peaking at the 4-hour time point with extended hypoxic exposure (levels gradually decreased; data not shown).

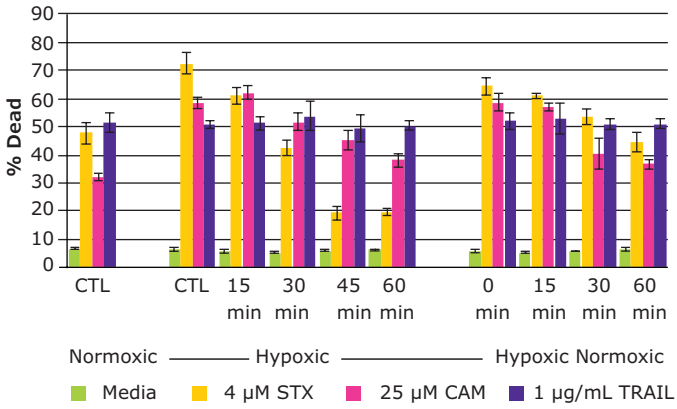
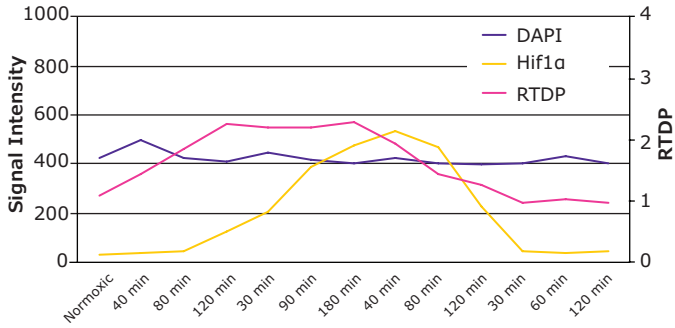


Figure 3. Chemoresistance is rapid, mitochondria-dependent, and reversible. Cell viability is measured based on SYTOX™ Green staining.



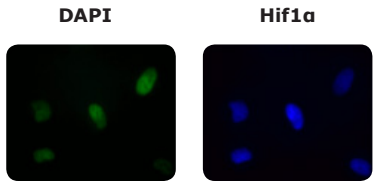


Figure 4. Chemoresistance changes parallel HIF1α protein levels. Data is presented as fluorescence intensity on a per cell basis (normalized). Total cells (DAPI); hypoxia-responsive fraction (inset images, 20X).

Hypoxic responses were differential amongst cancer cell lines **(Figure 5**). Eight cancer cell lines from diverse tissue origins and of different metastatic potential were preconditioned to hypoxia, with or without re-establishing standard oxygen levels, and exposed to STX. All cell lines except for HeLa showed reduced sensitivity to STX. However, there were significant differences in the rate of expression of the refractive phenotype. Reversibility was similarly variable, likely due to cell-specific differences in protein expression and turnover rates. Cells under prolonged hypoxic conditions underwent autophagic cell death (**Figure 6**). Under normoxic conditions, perfusing the culture chamber with caspase inhibitor zVADfmk (0.5 µM X 16 HR) inhibited STX-induced apoptosis. However, 3-methyladenine (3-MA, autophagy inhibitor via PI3-Kinase block) had no effect. Though short term hypoxia was generally protective, longer hypoxic exposure (24 hour) resulted in increased cell death. The mechanism for cell death may be governed by autophagy-based mechanisms, as prior exposure to 3-MA resulted in a significant reduction in the dead cell fraction.

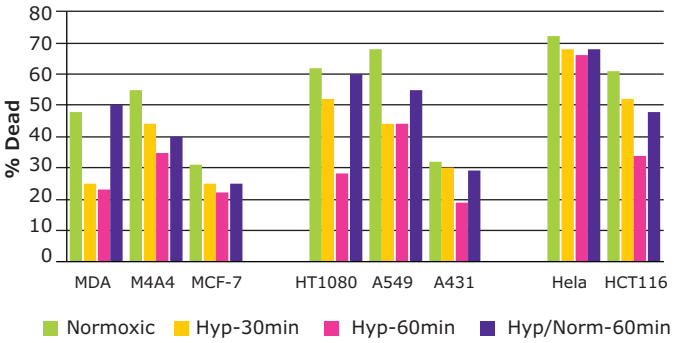


Figure 5. Cancer cell lines demonstrate differing hypoxic responses.

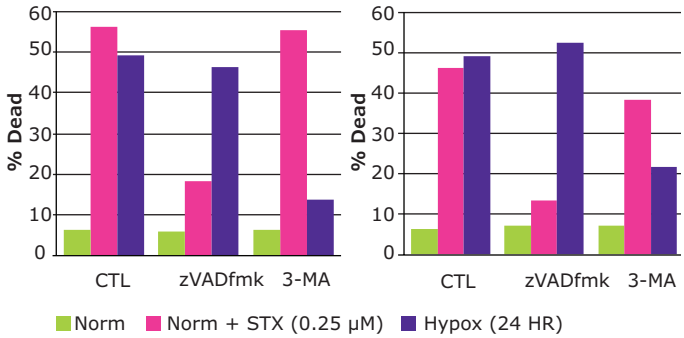


Figure 6. Prolonged hypoxia induces cell death via autophagy.

Hypoxia impacts metastatic potential – gradient plate invasion assays

Hypoxia in tumor cells has been linked to greater invasive capacity *in vitro*, and greater metastasis in human patients. Fluorescent labeling permits simultaneous analysis of two or more unique populations using the CellASIC ONIX2 System (**Figure 7**).

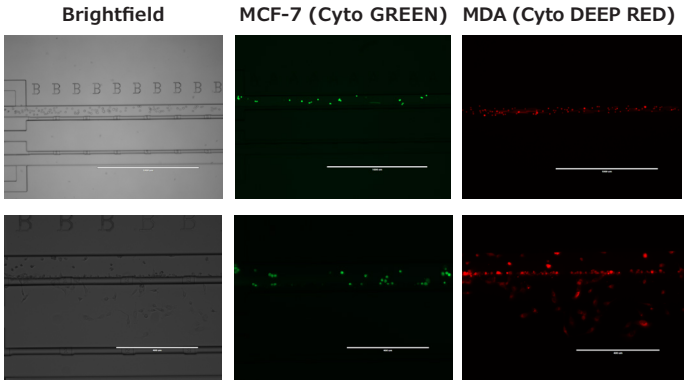


Figure 8. Invasion assays on the CellASIC® ONIX2 System. MDA MB231 cells (Cyto DEEP RED); MCF7 cells (Cyto GREEN).

As shown, highly metastatic MDA MB231 cells show significant penetration of the matrix compartment following 36 hour exposure to an FBS gradient (0-20%). By contrast, there is little directed movement of non-invasive MCF7 line. This experiment assessed the metastatic potential of four cancer cell lines in response to hypoxia, and demonstrated differential impact on invasive capacity (**Figure 8**).

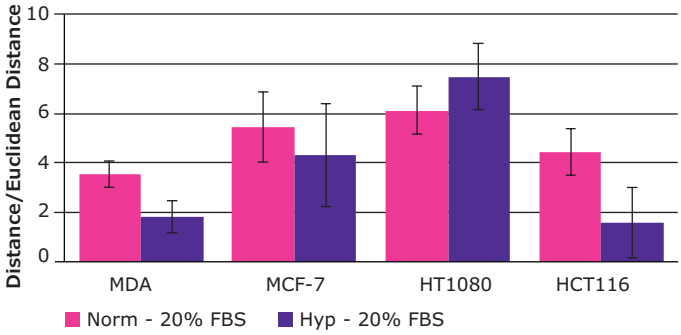


Figure 8. Hypoxia affects the invasive capacity of cancer cells. Directed cell movement is defined by the ratio calculated by the total distance moved divided by the Euclidean distance.

Discussion

Intermittent hypoxia in solid tumors is correlated with chemoresistance and poor clinical outcome. We studied the hypoxic responses of several cancer cells using the CellASIC® ONIX2 Microfluidic System and assay-optimized plates, which enable precise control over micro-environmental conditions, including gas and media content. We observed that hypoxia impacts numerous cell processes and functions including response to cytotoxic agents, mechanisms of cell death, and invasive capacity. The synergistic interplay between micro-culture and real-time imaging provides an automated framework for studying the onset of hypoxia, as well as the action of potential inhibitory therapeutics.

References

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