APPLICATION NOTE

# Inhibition of Hypoxic Tumor Cells using a Three-Dimensional Spheroid Model

## Comparison of PMT-based whole well detection with image-based cellular analysis

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## Cyto-ID® Hypoxia/Oxidative Stress Detection kit (ENZ-51042)

### Introduction

Discovering innovative cancer therapies requires a deep understanding of the heterogeneous microenvironment of solid tumors. The Warburg effect describes the metabolic shift from oxidative phosphorylation to aerobic glycolysis[1] that takes place in tumors, perhaps, even before the inevitable hypoxia that occurs as the tumor outgrows its insufficient and often irregular vasculature. The culmination of metabolic changes in cancer cells contributes significantly to increased metastasis and drug-resistance, aspects known to increase patient mortality[2,3]. Based on these observations, proteins that mediate metabolism and directly target hypoxic cells in primary, metastatic tumors are attractive targets for therapeutic intervention.

Spheroids, self-assembled microscale aggregates of cells, generated in hanging drop plates (HDPs) are a superior model of avascular microtumors. Because of their 3D structure, spheroids contain mass-transfer gradients of oxygen, nutrients, wastes, and therapeutic drugs that are highly comparable to what is observed in tumors within the human body. The metabolic gradients drive proliferation gradients and spheroids contain quiescent cells hypothesized to mimic drug resistant populations within tumors. Given sufficient cell numbers and time in culture, spheroids can develop hypoxic cores that can progress to necrosis, closely mimicking what is observed *in vivo*[4].

Here we show the ability to interrogate compounds that alter the hypoxic microenvironment of tumors, using spheroids grown in HDPs and a simple fluorescent dye that specifically measures the activity of hypoxic cells. For the initial test we chose an inhibitor of carbonic anhydrase IX (CA IX), a hypoxia inducible factor 1$\alpha$ (HIF-1$\alpha$)-regulated protein that functions to maintain intracellular pH[2]. This family of inhibitors has been shown to reduce cancer cell growth and tumor metastasis[5]. Imaging of spheroids in the hanging drop, following treatment, was performed to observe the inhibitory effects of the compound. The results demonstrate the validity of the 3D cell model, and the use of this method to predict potential downstream anti-metastatic effects of lead molecules.

## Materials and Methods

### Materials

#### Cells

Colorectal carcinoma HCT116 cells (Catalog No. CCL-247) were obtained from ATCC (Manassas, VA, USA). The cells were propagated in McCoy's 5A (Modified) Medium, HEPES plus Fetal Bovine Serum, 10% and Pen-Strep, 1x.

RFP-expressing human neonatal dermal fibroblasts (Catalog No. cAP-0008RFP) were purchased from Angio-Proteomie (Boston, MA, USA). The cells were propagated in Dulbecco's Modified Eagle Medium (DMEM), high glucose, pyruvate plus Fetal Bovine Serum, 10% and Pen-Strep, 1x.

#### Reagents

Hypoxia Red Detection Reagent, part of the Cyto-ID® Hypoxia/Oxidative Stress Detection Kit (Catalog No. ENZ-51042) was provided by Enzo Life Sciences (Farmingdale, NY). The reagent is a weakly-fluorescent compound containing a nitro ($NO_2$) moiety. Due to nitroreductase activity present in hypoxic cells, the nitro group is converted to hydroxylamine and amino groups. The original molecule then degrades releasing the fluorescent probe. Carbonic anhydrase (CA) inhibitor U 104 was used to inhibit hypoxia.

#### 96-Well Hanging Drop Plates

Perfecta3D® 96-Well Hanging Drop Plates (Catalog No. HDP-1096) were provided by 3D Biomatrix, Inc. (Ann Arbor, MI).

#### Instrumentation

The MultiFlo FX Microplate Dispenser was used to dispense cells and medium to the wells of the Hanging Drop Plates.

The Cytation(TM) 3 combines automated digital microscopy and conventional multi-mode microplate detection providing rich phenotypic cellular information and well-based quantitative data. The instrument was used to image the 3D spheroids in the hanging drops following addition of the Hypoxia Red Detection Reagent. The Cellular Analysis feature of the Gen5™ Data Analysis Software was used to detect the fluorescent signal from the Hypoxia Red Detection Reagent solely within the spheroids using optimized parameters.

### 3D Cell Culture Components

#### *Perfecta3D 96-Well Hanging Drop Plates*

3D Biomatrix's HDPs facilitate 3D spheroid formation in 96- or 384-well formats. A drop of cell suspension is pipetted into the top of each well, and the plate geometry causes the drop to hang stably below the well. Spheroid diameter can be controlled with the type and number of cells added. Access holes at the top of each well allow for media exchange and the addition of compounds, reagents or additional cells or spheroids to establish co-cultures. Without contact with any surfaces or matrices, cells aggregate to form one spheroid per well.

## Methods

### Cell Preparation and Automated Dispensing into Hanging Drop Plates

HCT116 and fibroblast cells were harvested and diluted in HCT116 media to concentrations of $6.25x10^4$ cells/mL. Prior to dispensing, the HDP plate and tray reservoirs were filled with 3 and 5 mL of sterile Dulbecco's phosphate buffered saline (DPBS), respectively. The cells were combined together and a volume of 40 µL was then dispensed by the MultiFlo FX to create spheroids containing 5000 cells per spheroid and equal numbers of each cell type. Following dispensing, the plate assembly was placed at $37^oC/5\%$ $CO_2$.

### Image-Based Spheroid Formation Monitoring

Spheroid formation was monitored every 24 hours. The HDP assembly was placed into the Cytation 3, and manual imaging was performed using a 4x objective to determine the state of aggregation. The imager can focus through the clear tray below the hanging drops, part of the complete plate assembly; therefore it was not necessary to remove the tray before placing the plate onto the stage.

### CA Inhibitor Dosing

Upon completion of cell aggregation, media was removed from the hanging drops and replaced with media containing U 104 at concentrations of 0, 100, 1000, and 10,000 nM. The procedure involved slow removal of 10 µL of media from the top of each access hole, followed by a 10 µL addition of media with compound, which was repeated 5-6X to ensure a complete media exchange. Dosing was repeated on a daily basis over the two-week incubation period. .

### Hypoxia Reagent Incubation Optimization

The hypoxia detection reagent was diluted to a 5X concentration in HCT116 media. A volume of 10 µL was then added to the appropriate wells containing formed spheroids to create a final 1X concentration. The reagent was then incubated with the spheroids for either 2, 4, or 6 hours at $37^oC/5\%$ $CO_2$. Following incubation, reagent-containing media was washed out with DPBS using the same procedure previously explained for CA inhibitor dosing. Fluorescent imaging was then performed using the Texas Red imaging filter cube to determine which incubation time yielded the greatest reagent penetration into the spheroid, and therefore lowest background.

### Analysis of Spheroid Hypoxia

Hypoxia detection reagent (5X) was added to appropriate wells following 1, 4, 7, and 14 days of compound treatment, incubated for the optimized time period, and then washed with DPBS as previously described. Fluorescent and brightfield imaging were then performed using a 4x objective and consistent LED excitation, integration time, and gain settings to ensure that changes in hypoxia levels were captured.

Cellular analysis was performed using the Gen5 software on the 4x images of the spheroid captured. This was done in order to analyze only the fluorescent signal from the cells in the spheroid itself, and to ignore all other portions of the image. Table 1 describes the parameters used.

| Cellular Analysis Parameters | |
|---|---|
| Detection Channel | Brightfield |
| Threshold | 10,000 RFU |
| Min. Object Size | 500 µm |
| Max. Object Size | 1000 µm |
| Bright objects on a dark background | Unchecked |
| Split touching objects | Checked |
| **Advanced Options** | |
| Evaluate Background On | 5% of Lowest Pixels |
| Image Smoothing Strength | 0 |
| Background Flattening Size | Auto |

**Table 1. 4x Image Cellular Analysis Parameters.**

The brightfield image was used as the detection channel in order to properly define the boundaries of the spheroid. The fluorescence from the hypoxia reagent within the spheroid boundaries, captured with the Texas Red channel, was then used for analysis.

## Results and Discussion

### Optimization of Hypoxia Red Reagent/Spheroid Incubation

The fluorescent signal from the hypoxia dye was assessed within the spheroid after a 2 hour incubation. As seen from the image in Figure 1A, a relatively small concentration of reagent was integrated into the cells after two hours. Therefore the required image exposure to see the signal within the spheroid was set to near maximum. This led to high background fluorescence, making accurate cellular analysis difficult to accomplish.

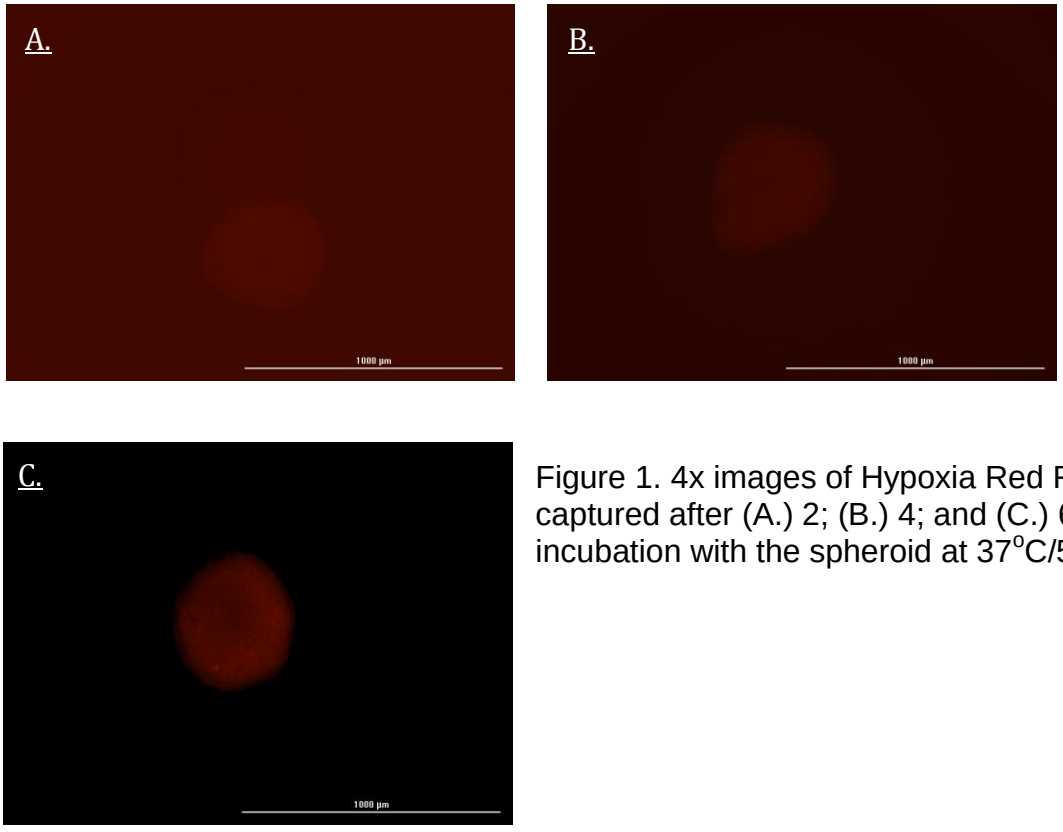


Figure 1. 4x images of Hypoxia Red Reagent signal captured after (A.) 2; (B.) 4; and (C.) 6 hours of incubation with the spheroid at 37$^{o}$C/5% $CO_2$.

Examination of the image containing reagent incubated with the spheroid for four hours (Figure 1B) reveals increased reagent integration. However, exposure settings still generated relatively high background signal. Finally, when using a 6-hour incubation time, a sufficient amount of reagent was integrated, which allowed for exposure settings that yielded low background fluorescence while still giving a bright signal from the reagent within the spheroid. This incubation time was used for all subsequent experiments.

### Confirmation of Fibroblast RFP and Hypoxia Red Reagent Signal

Human fibroblasts have been shown to play an important role in determining tumor cell behavior, as well as conditioning the tumor microenvironment[6, 7]. Therefore they were also included in this study to create a suitable 3D spheroid model. As RFP-expressing fibroblasts were used, imaging was also performed of spheroids following initial cell aggregation using the brightfield and RFP imaging filter cubes to confirm that the red fluorescent RFP signal would not artificially contribute to the signal captured with the Texas Red Imaging Cube.

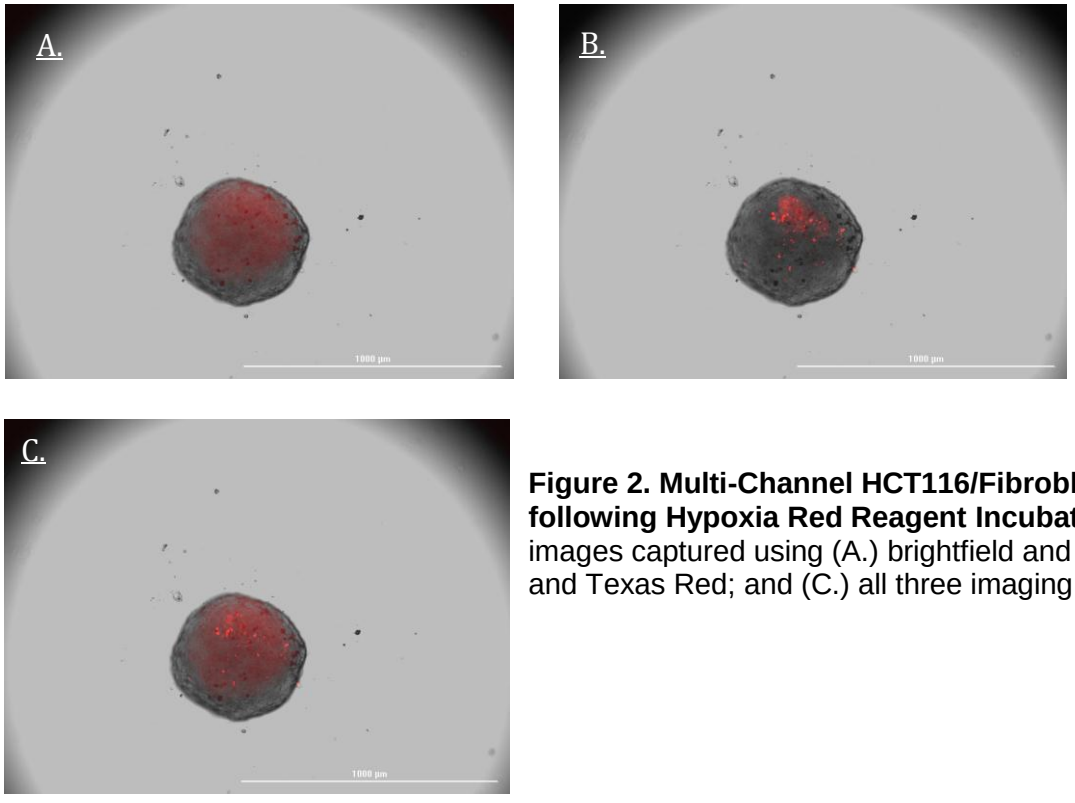


**Figure 2. Multi-Channel HCT116/Fibroblast Images following Hypoxia Red Reagent Incubation.** Overlaid 4x images captured using (A.) brightfield and RFP; (B.) brightfield and Texas Red; and (C.) all three imaging filter cubes.

Figure 2A exhibits overlaid brightfield and RFP images showing total cells as well as fibroblasts expressing RFP. Figure 2B displays the same brightfield image in addition to the signal from the Hypoxia Red Reagent captured using the Texas Red channel. When comparing the red fluorescent signal in Figure 2A and B, as well as the complete overlaid image in Figure 2C it is evident that RFP fluorescence is not captured when using the Texas Red Imaging Cube.

## Analysis of Hypoxia Inhibition

Carbonic anhydrase IX (CA IX) is a hypoxia-inducible protein that promotes tumor cell survival and metastatic cell invasion[5]. Therapeutic inhibition of CA IX has been shown to decrease tumor growth and metastasis. The Hypoxia Red Reagent relies on the nitroreductase properties of active hypoxic cells; as these cells lose functionality less reagent is converted leading to a decrease in fluorescence. The ability of U 104 to affect nitroreductase activity was assessed over a two week period. The 4x images in Figure 3 illustrate the inhibitory effect of the compound at a 10 µM concentration compared to the negative control.

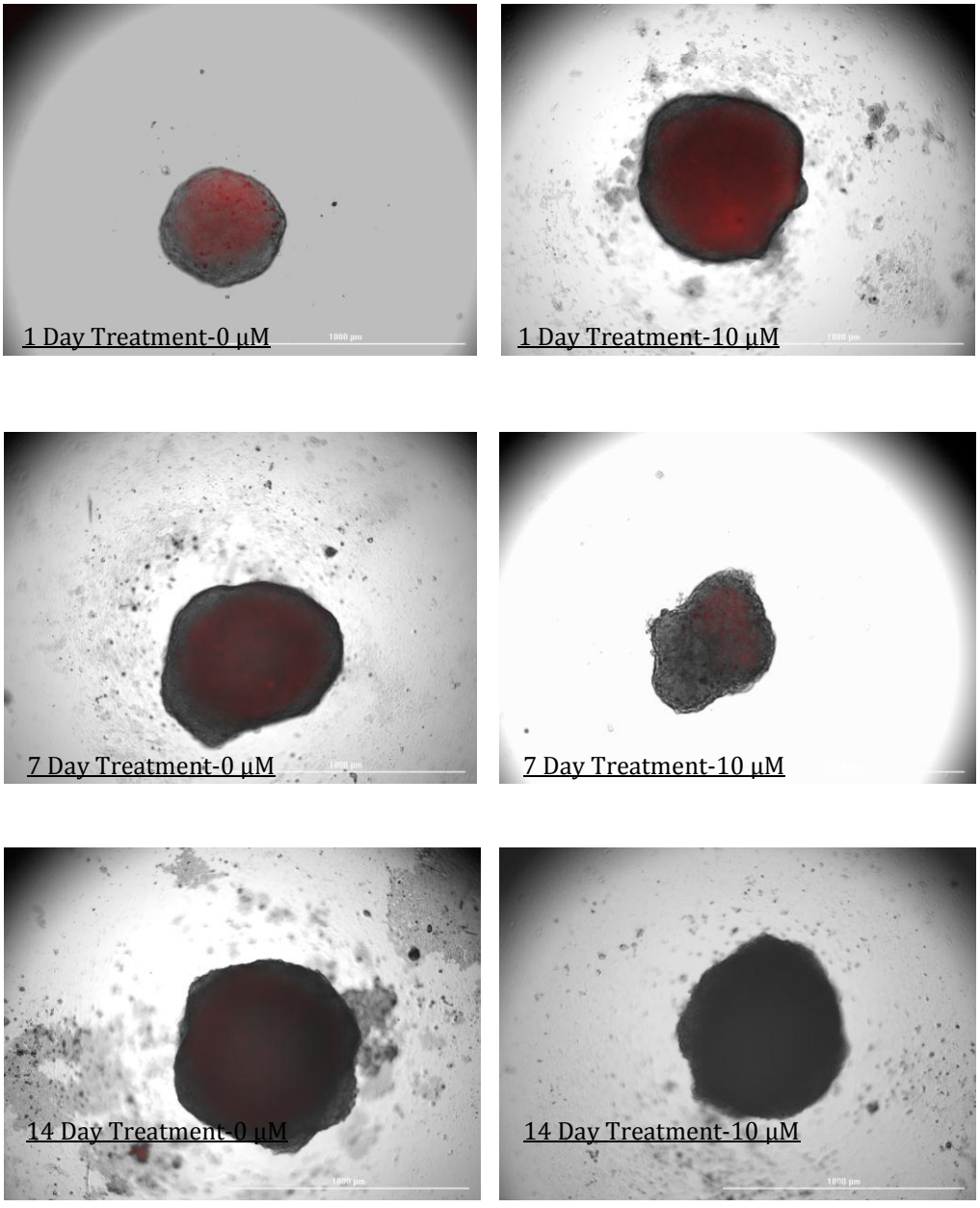


**Figure 3.** 4x brightfield and Texas Red overlaid images of HCT116/fibroblast spheroids, following Hypoxia Red Reagent addition and incubation.

Using the Cellular Analysis parameters outlined in Table 1 with the 4x brightfield and Texas Red images, the signal from the Hypoxia Red Reagent was evaluated within each spheroid analyzed. Figure 4 exhibits the cellular masks that were drawn around the spheroids evaluated after a 14 day U 104 treatment.

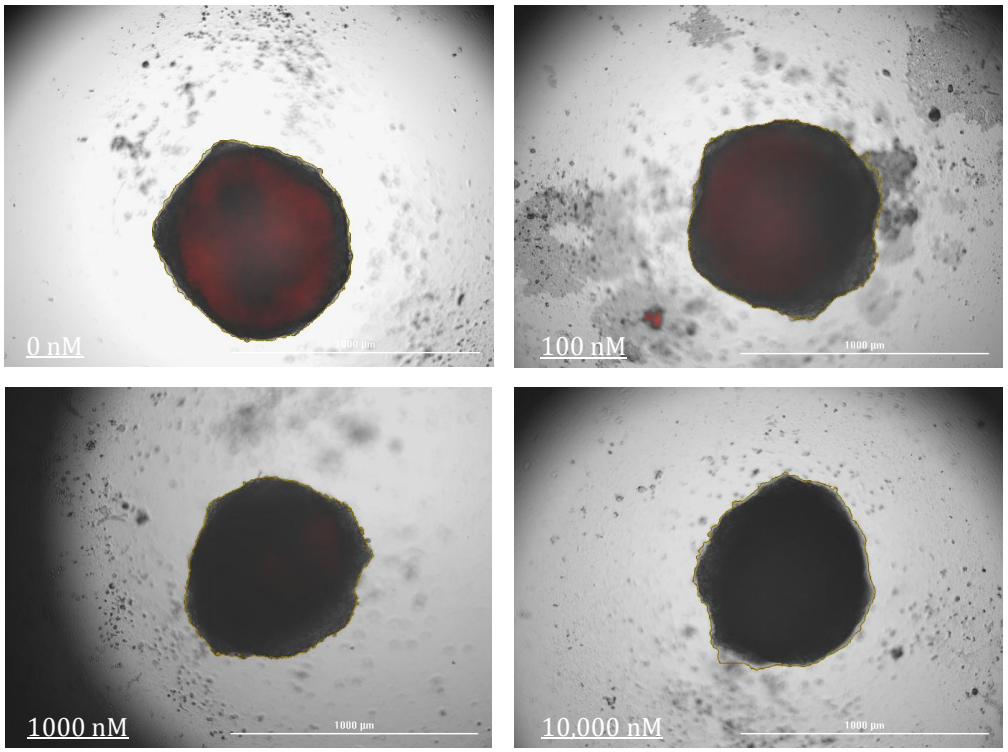


**Figure 4.** Object masks drawn by Gen5 around HCT116/fibroblast spheroids following 14 day U 104 treatment, using the criteria outlined in Table 1.

The raw fluorescent signal generated from active hypoxic cells within each spheroid was plotted for each compound concentration tested over the two-week dosing period (Figure 5A). Normalization was then completed by comparison of fluorescent units from subsequent day analyses to values from the initial analysis after a one day incubation, and expressed as a percentage (Figure 5B).

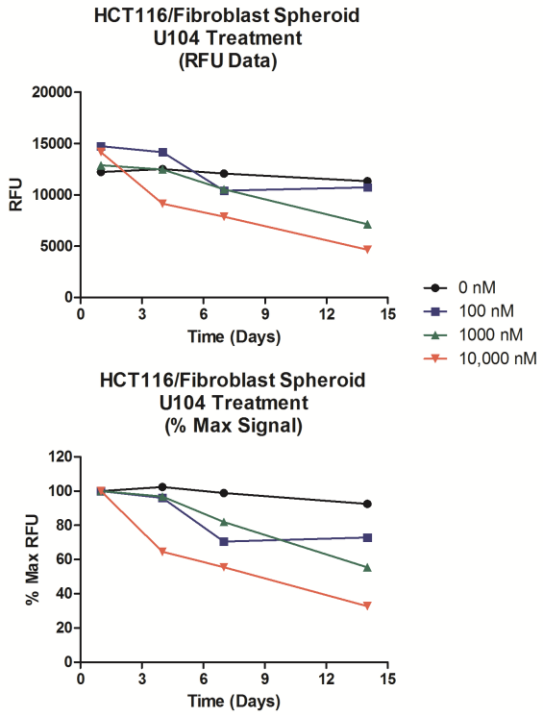


**Figure 5. U 104 Induced Reduction of Hypoxic Cell Nitroreductase Activity.** (A.) Mean raw fluorescence values from Hypoxia Red Reagent using a 4x objective and the Texas Red Imaging Cube; (B.) Normalization of fluorescent signal calculated by the following formula: (Mean RFUDay X / Mean RFUDay 1).

Results from Figure 5 demonstrate that the carbonic anhydrase inhibitor U 104 specifically affects hypoxic tumor cells, as witnessed by the dose-dependent decrease in nitroreductase activity over the 14-day dosing period. We hypothesize that the reduction in fluorescent signal is due to increased cell death of hypoxic cells. This agrees with what has been shown previously in the literature, that therapeutic treatment of tumor cells by inhibition of CA IX is accomplished in part by decreasing the ability of cells to adapt to the low extracellular pH found in hypoxic regions of primary tumors[7].

## Conclusions

We have demonstrated that cancer cell/fibroblast co-cultured spheroids can be used to successfully observe the effects of test compounds, such as CA IX inhibitors, on hypoxic tumor cells. Cells and media can be rapidly and efficiently dispensed to the Perfecta 3D Hanging Drop Plates, where spheroid formation is accomplished and can be monitored via imaging in real time. Detection of the activity of hypoxic cells can also be easily completed with the incorporation of the Hypoxia Red Reagent and experimental imaging taking place within the hanging drop. The combination can provide initial information regarding the potential final effect of test compounds on tumor growth and metastatic activity.

## References

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