APPLICATION NOTE

PMT and Image-Based Analysis of Hypoxia Induction using a 3D Spheroid Model

Monochromator, Image, and Cellular Analysis of Kinetic Hypoxia Induction in a 3D Liver Microtissue

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

Introduction

Cell-based assays provide a wealth of drug screening information compared to their biochemical counterparts, and at a fraction of the cost and time seen with traditional animal models. It is estimated that half of the screens performed for target validation and ADME-Tox now incorporate a cell-based format[1]. The evolution into live cell formats provides valuable data that may be missed in a single time point, lytic assay. In addition to a deeper understanding of cellular mechanics and responses with the fact that cells are still intact during the detection process, live cell assays offer increased flexibility through kinetic, or multiplexed analyses. A downfall of both these assay types is that cells grown on solid, flat substrates do not display the same morphology, and may not behave in the same manner, as those grown in vivo[1].

Unlike ex vivo 2D cultured cells, those grown in vivo interact with nearby cells and the extracellular matrix (ECM) to form complex communication networks that control a number of cellular processes[2]. New 3D culture methods encourage cells to aggregate into clusters, thereby forming vital communication networks, and more closely mimicking in vivo structures. As these cell clusters, or spheroids, are quite small relative to the entire area of a microplate well, accurate pmt-based detection may be difficult. Imaging the spheroids, and incorporating cellular analysis instead of whole well analysis, may offer greater sensitivity.

Here, we investigate the impact of exposing cells cultured into a 3D microtissue to hypoxic atmospheric conditions. Kinetic monitoring was carried out by performing whole well monochromator-based reading and imaging over an 11 hour time period. Analysis of changes in whole-well and whole-image fluorescence intensity values, as well as changes specifically from cells making up the spheroid alone, via cellular analysis, demonstrate how the latter allows for a more accurate account of the final effect.

Materials and Methods

Materials

Cells

3D InSight™ Human Liver Microtissues composed of primary human hepatocytes were purchased from InSphero, Inc. (Cambridge, MA)

Reagents

Hoechst 33342 was used as a nuclear stain while the Hypoxia Red Detection Reagent, part of the Cyto-ID® Hypoxia/Oxidative Stress Detection Kit (Catalog No. ENZ-51042) was donated by Enzo Life Sciences (Farmingdale, NY).

Instrumentation
The Cytation™ 3 Cell Imaging Multi-Mode Reader was used to perform kinetic monochromator-based microplate reading, as well as imaging of the microtissues. The instrument maintained 37°C/8% O_2 atmospheric conditions during the monitoring process through the use of software settings and a gas control module.

Methods

Cell Propagation
Prior to performing the experiment, medium was exchanged in the 96-well GravityTRAP™ plates containing the 3D liver microtissues every two to three days.

Hypoxia Study Cell Preparation
Medium was removed from the 96-well GravityTRAP™ plate and replaced with medium containing Hoechst 33342. The plate was placed back into the tissue culture incubator for 10 minutes. Medium was once again removed and replaced with fresh medium containing 500 nM Cyto-ID® Hypoxia reagent. The medium and microtissue were then transferred into an empty GravityTRAP™ plate to perform the experiment.

Cell/Spheroid Imaging
Preceding initial cell imaging, Cytation 3's temperature control was set to 37°C, and the gas control module was set to 8% O2. Nitrogen was sparged into the instrument to maintain the 8% O_2 level. A Discontinuous Kinetic Procedure was used to perform the microplate reading and imaging once per hour over an 11 hour time period. Microplate reads were completed by the monochromator-based detection system using Ex. 596 nm/Em. 670 nm settings. Imaging was carried out with the DAPI and Texas Red imaging filter cubes to capture the signal from the Hoechst 33342 and Cyto-ID® Hypoxia Reagent, respectively, using 4x and 10x magnification. Each cube consists of the appropriate colored LED excitation source, as well as excitation and emission filters, and dichroic mirrors. Image capture settings were set manually prior to time 0 imaging to ensure signal from the hypoxia reagent could be captured above background, while also ensuring that pixels in subsequent images would not be over exposed.

Hypoxia Red Detection Reagent Signal Analysis
Whole Well Monochromator-Based Signal Detection:
Total signal intensity from the hypoxia reagent was captured from the entire well via the PMT using the monochromator excitation and emission settings previously described.

Whole Image LED/CCD-Based Signal Detection:
Mean signal intensity from the hypoxia reagent was captured from the entire image, using either 4x or 10x objectives. LED excitation and a 16-bit CCD camera were incorporated to increase the excitation signal, and also provide a more sensitive method of signal detection from the cells.

Whole Spheroid LED/CCD-Based Signal Detection:
Cellular analysis was performed using the Gen5 software on the 10x images of the spheroid captured as previously explained. This was done in order to analyze only the fluorescent signal from the cells in the spheroid itself, and ignore all other portions of the image. Table 1 describes the parameters used.

Cellular Analysis Parameters	
Threshold	500 RFU
Min. Object Size	150 µm
Max. Object Size	300 µm
Bright objects on a dark background	
Split touching objects	
Advanced Options	
Evaluate Background On	5 % of Lowest Pixels
Image Smoothing Strength	0
Background Flattening Size	500 µm

Table 1. 10x Image Cellular Analysis Parameters.

Results and Discussion

The fluorescent signal from kinetic microplate reads as well as from 4x and 10x imaging was used to analyze the effect of exposing the liver microtissue to low oxygen (hypoxic) conditions. The images captured using a 10x objective are exhibited in Figure 1.

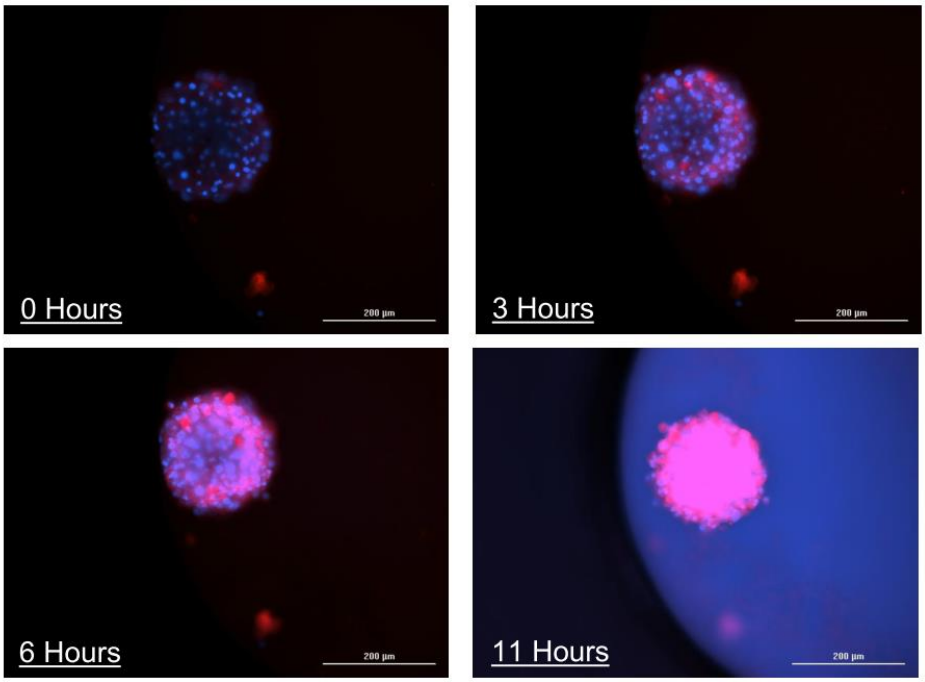

Figure 1. 10x DAPI and Texas Red overlaid images of Hoechst 33342 stained cell nuclei and Hypoxia Red Reagent, respectively. Images captured after liver microtissue was incubated for 0, 3, 6, and 11 hours at 37^{o}C/8% O_2.

Hypoxia Induction Monitoring using Monochromator-Based Detection

Using a PMT gain of 80 and the excitation and emission wavelengths previously described, the hypoxia red reagent fluorescent signal was quantified from the entire well being monitored.

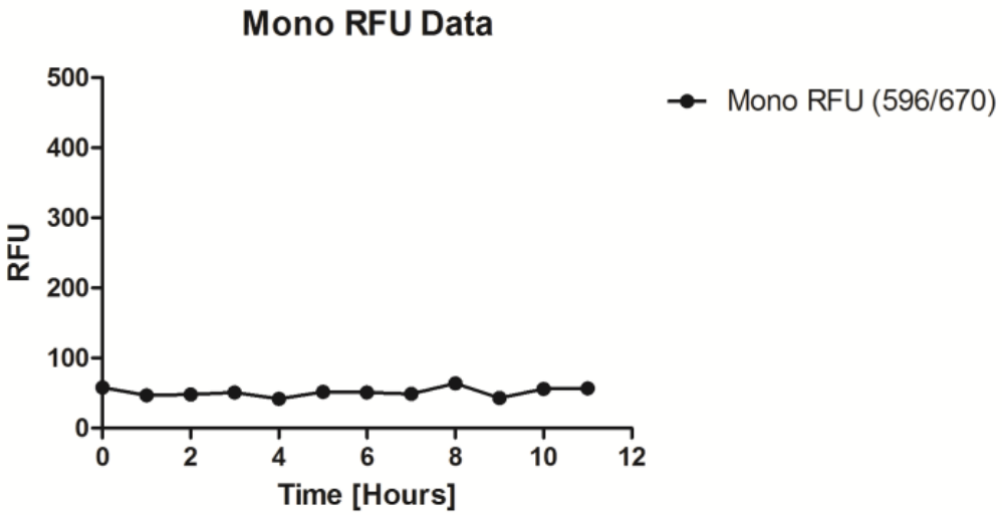

Figure 2. Kinetic fluorescent signal from Hypoxia Red Reagent using monochromator based detection.

As can be seen by the results in Figure 2, the fluorescent values do not change over the 11 hour time period using this type of detection. The reason for this phenomenon is due to the fact that the area of a liver microtissue is approximately 1000X smaller than the area of the well of a 96-well microtiter plate (Figure 3). The latter of which is typically quantified by PMT-based detection. Therefore any change in signal coming from the microtissue will many times be imperceptible, and the true results of the test may go undetected.

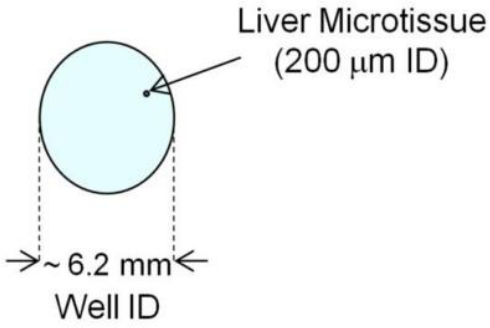

Figure 3. Size comparison of typical liver microtissue and 96-well microtiter plate well.

Image-Based Analysis of Hypoxia Induction

The hypoxia red reagent fluorescent signal was also quantified using 4x and 10x imaging, and the Image Statistics analysis capabilities of the Gen5 Data Analysis Software. The mean fluorescence from the hypoxia reagent captured in the entire image using the Texas Red imaging filter cube was calculated. In this way only the signal from a smaller, more focused portion of the well containing the liver microtissue is measured. Figure 4 demonstrates the portion of the well imaged and used for analysis using the 4x and 10x objectives.

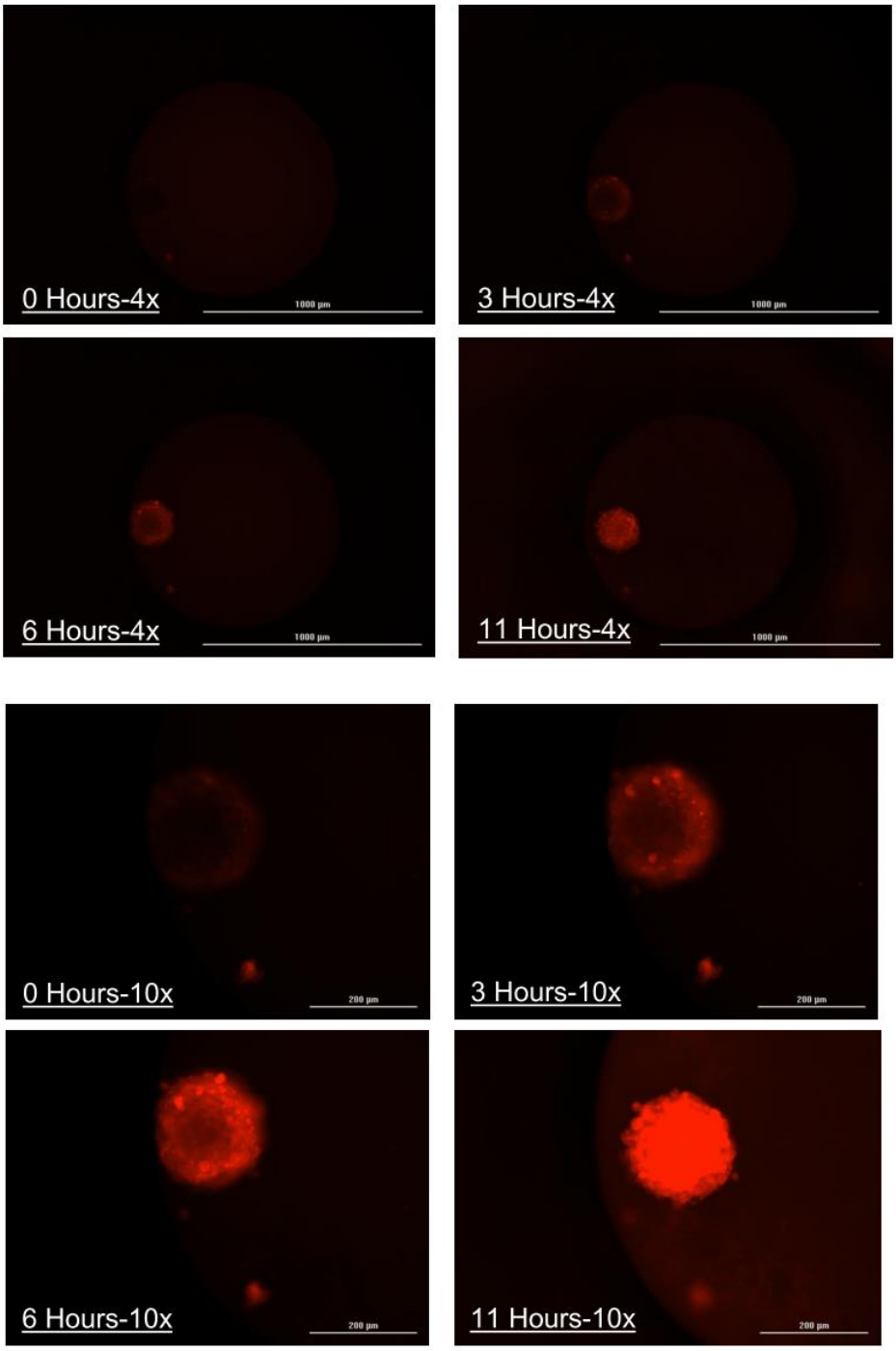

Figure 4. 4x and 10x kinetic images captured of liver microtissue using the Texas Red imaging filter cube.

From the mean fluorescence values calculated from 4x (Figure 5A) and 10x (Figure 5B) images, it is evident that the combination of LED excitation and CCD-based quantification, as well as being able to focus on a smaller portion of the well containing the sample of interest, creates a more robust analysis of the change in signal from the hypoxia reagent. This is especially true when focusing on the results from the 10x image analysis, where a steady increase in fluorescent signal is seen at each time point.

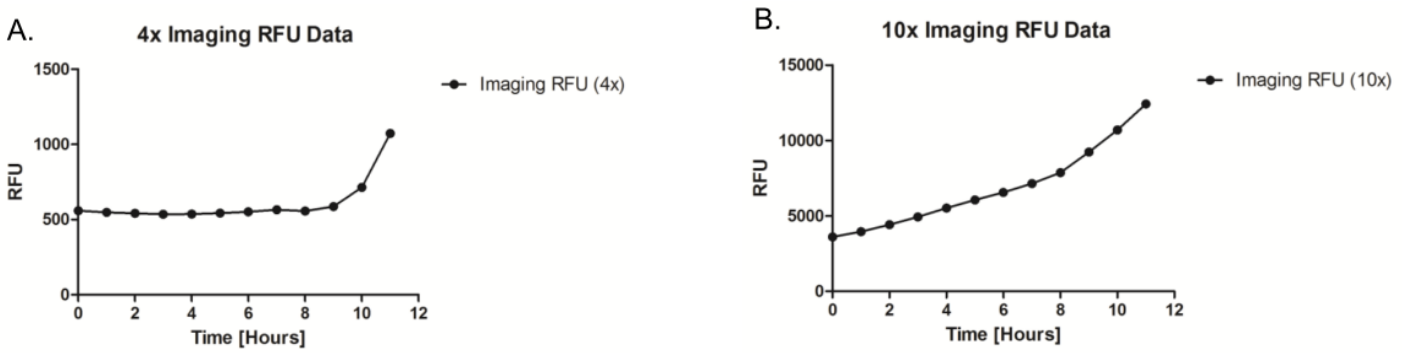

Figure 5. Hypoxia Red Reagent mean fluorescence values calculated from (A.) 4x and (B.) 10x kinetic images.

Cellular Analysis of Hypoxia Induction

Using Gen5, a Cellular Analysis was also performed using the 10x images captured with the Texas Red imaging filter cube. By setting the parameters outlined in Table 1, the software is able to define a mask around the liver microtissue (Figure 6).

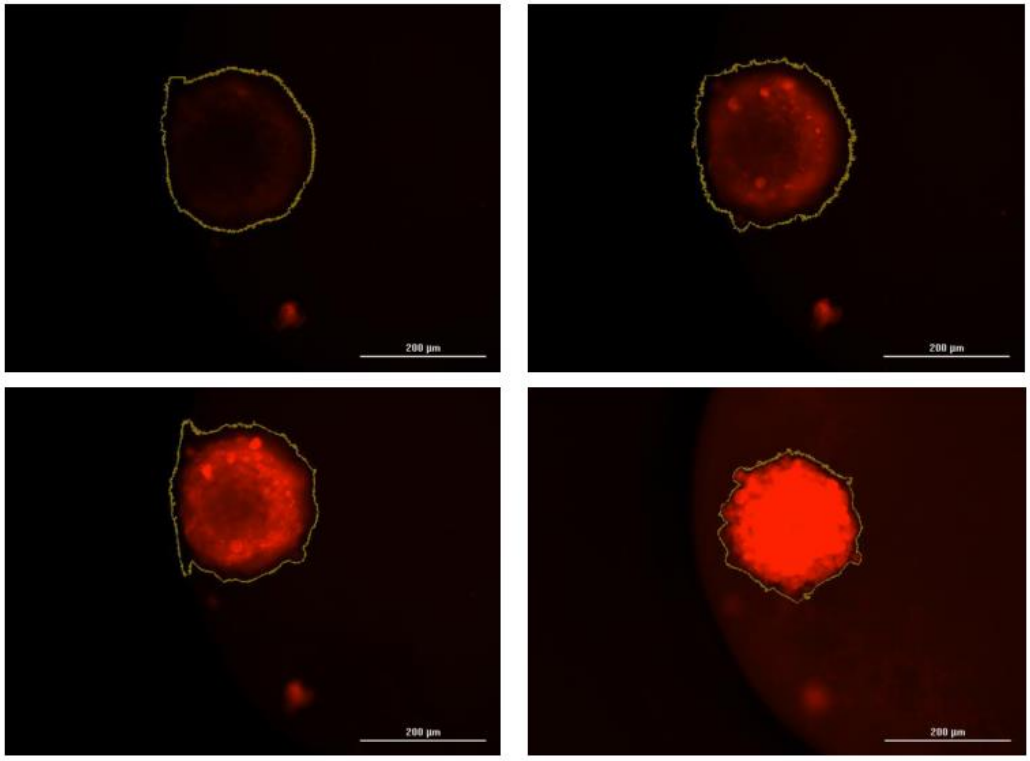

Figure 6. Object masks drawn by Gen5 around liver microtissues using the criteria outlined in Table 1.

This allows for signal quantification from the entire object as a whole, instead of from each individual cell. Using this technique, only the fluorescence within the drawn mask is calculated, while the rest of the image is ignored. Due to this fact, a large change in fluorescent signal is seen during the incubation period (Figure 7).

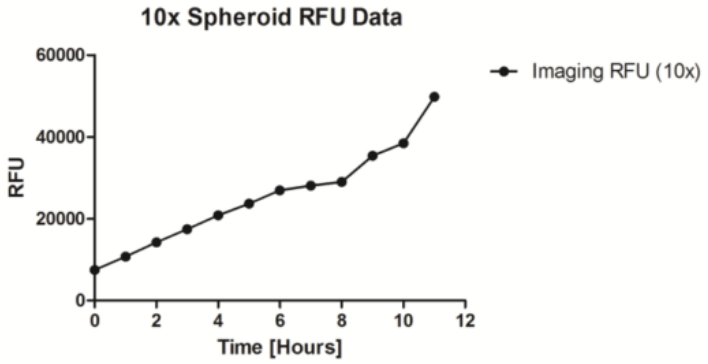

Figure 7. Hypoxia Red Reagent mean fluorescence values calculated from Cellular Analysis of 10x Texas Red images.

When performing a fold change analysis using the fluorescence values calculated with all three methods of signal quantification (Figure 8), it can be observed that the cellular analysis method creates the largest change in fluorescent signal over time, and therefore the most robust manner in which to evaluate the results from this type of experiment.

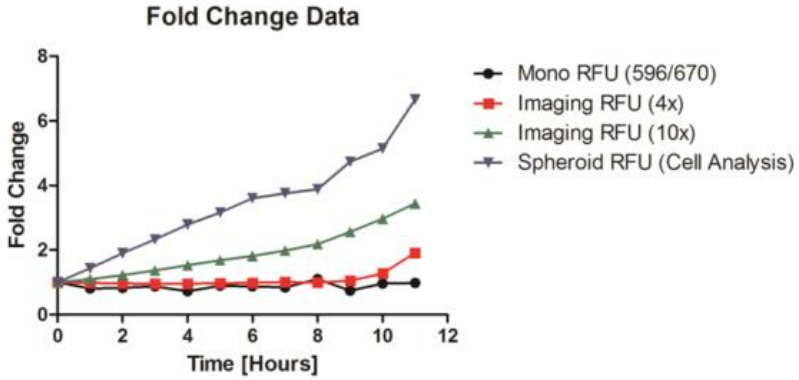

Figure 8. Hypoxia Red Reagent Fluorescence Signal Fold Change Analysis. Fold change calculated by the following formula: (Mean RFUTime X / Mean RFUTime 0).

Conclusions

The results presented here illustrate how the Cyto-ID® Hypoxia Red Detection Reagent, as well as the ability to perform kinetic reading and imaging with the Cytation 3 while maintaining constant appropriate low oxygen conditions through the incorporation of the gas control module, can be combined to easily and accurately monitor hypoxia induction in a liver microtissue cell model. Furthermore, a comparison of the various available methods for fluorescent signal detection and analysis demonstrate how limiting signal quantification specifically to the object of interest can create the most robust manner in which to perform experimental evaluation. Cellular analysis can also allow crucial phenomenon to be seen that may otherwise go undetected.

References

1. Zang, R.; Li, D.; Tang, I.; Wang, J.; Yang, S. Cell-Based Assays in High-Throughput Screening for Drug Discovery. J. Biotech. Well. Indus. 2012, 1, 31-51.
2. Bissel, M.J.; Radisky, D.C.; Rizki, A.; Weaver, V.M.; Petersen, O.W. The organizing principle: microenvironment influences in the normal and malignant breast. Differentiation 2002, 70, 537–546.