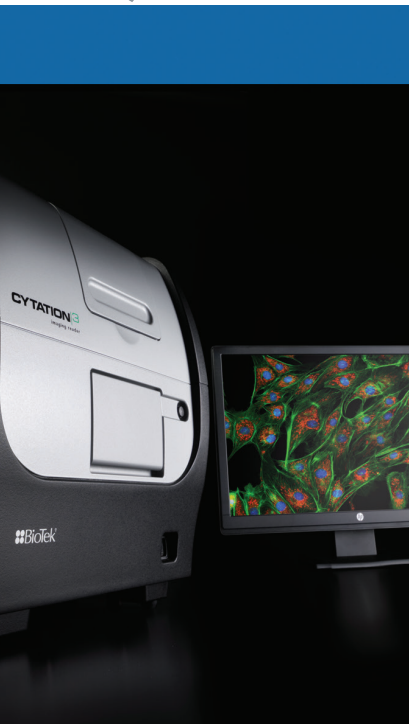


Live Cell Hypoxia Determination in a 3D Spheroid Model

Comparison of PMT-based Whole Well Detection with Image-based Cellular Analysis



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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 format¹. 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 processes². 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 and information.

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 (Catalog No. H3570) was purchased from Life Technologies (Carlsbad, CA), 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 and 8% O₂ 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.

Key Words:

3D Cell Culture

Spheroid

Hanging Drop

Fluorescence Imaging

Liver Microtissue

Hypoxia

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% O₂ 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. Figure 1 exhibits the images captured using the 10x objective. Note the growth of the red hypoxia signal that eventually overwhelms the Hoechst signal. By 11 hours, it appears that the integrity of the plasma and nuclear membranes of the cells comprising the spheroid have ruptured releasing labeled DNA from the nucleus into the bulk solution.

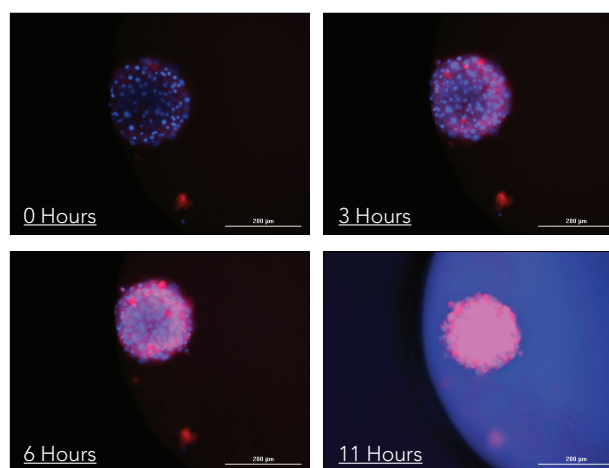


Figure 1. 10x overlaid images of Hoechst 33342 stained cell nuclei and Hypoxia Red Reagent, respectively. Images captured after liver microtissue was incubated for 0, 3, 6, and 11 hours at 37 °C/8% O₂.

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.

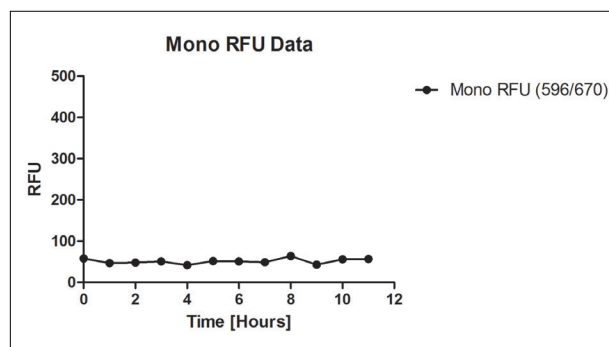


Figure 2. Kinetic fluorescent signal from Hypoxia Red Reagent using monochromator-based detection.

As can be seen by the results in Figure 2, the fluorescent values show no significant change over the 11 hour time period. The reason for this phenomenon is 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). PMT-based optics are designed to capture as much light from the well as possible, thus for live cell microtissue analysis, these optics collect largely background as evident from the temporal response in Fig 2.

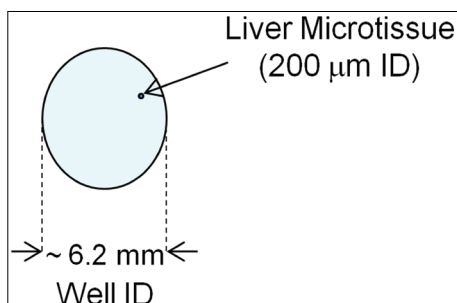


Figure 3. Relative size comparison of typical liver microtissue and 96-well microtiter plate well.

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, reducing the amount of background collected. Figure 4 demonstrates the portion of the well imaged and used for analysis using the 4x and 10x objectives.

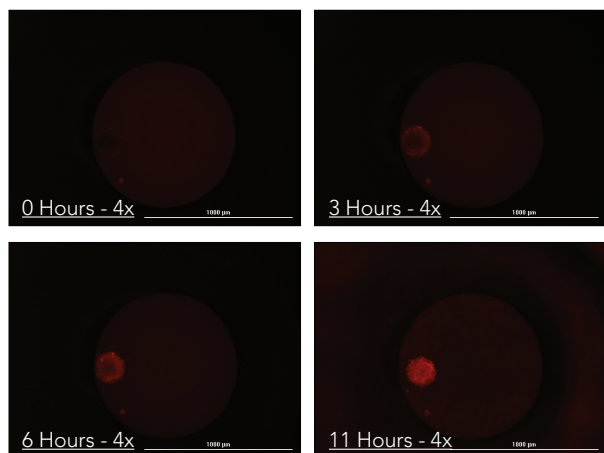


Figure 4A. 4x kinetic images captured of liver microtissue using the Texas Red imaging filter cube.

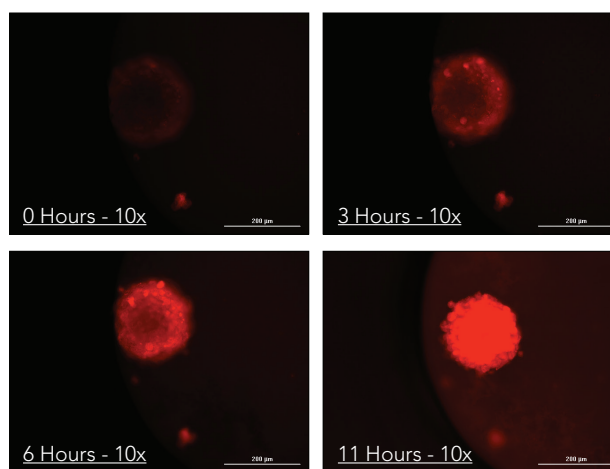


Figure 4B. 10x kinetic images captured of liver microtissue using the Texas Red imaging filter cube.

From the mean fluorescence values calculated from 4x and 10x images (Figure 5), 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.

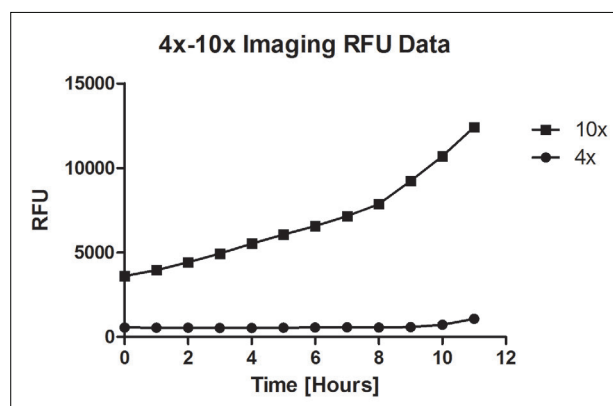


Figure 5. Hypoxia Red Reagent mean fluorescence values calculated from 4x and 10x kinetic images.

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).

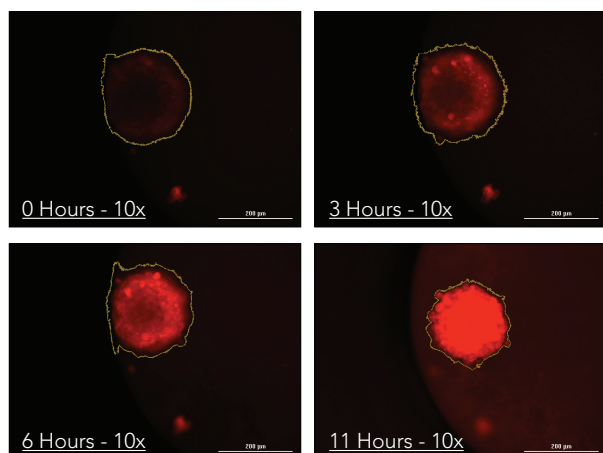


Figure 6. Object masks drawn by Gen5 around liver microtissues using the criteria outlined in Table 1.

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 consisting of background signal is ignored. Due to this fact, a large change in fluorescent signal is seen during the incubation period (Figure 7).

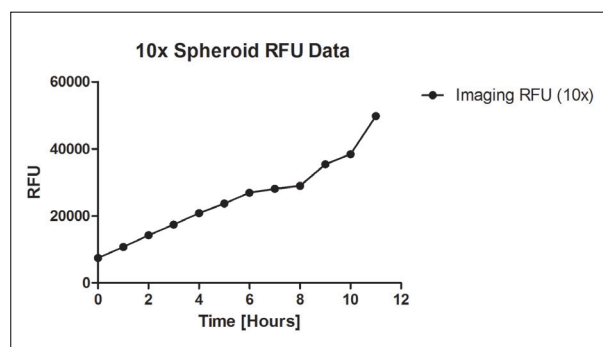


Figure 7. Hypoxia Red Reagent mean fluorescence values calculated from Cellular Analysis of 10x Texas Red images.

When performing a fold change analysis using the fluorescence values calculated with all four 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.

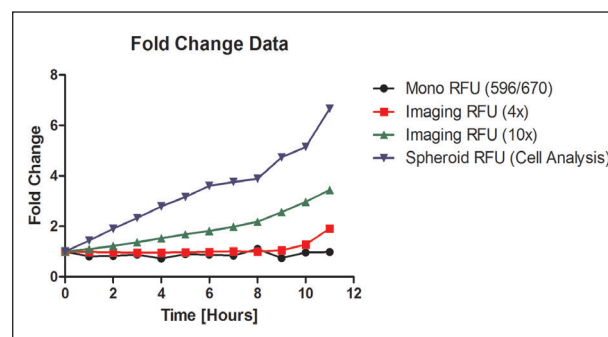


Figure 8. Hypoxia Red Reagent Fluorescence Signal Fold Change Analysis. Fold change calculated by the following formula: $(\text{Mean RFU}_{\text{Time X}} / \text{Mean RFU}_{\text{Time 0}})$.

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 highest signal to background ratio and thus greatest response.

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.

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