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ANALYSIS OF CELLULAR PROCESSES WITHIN 3D CELL MODELS USING FLUORESCENCE MICROSCOPY

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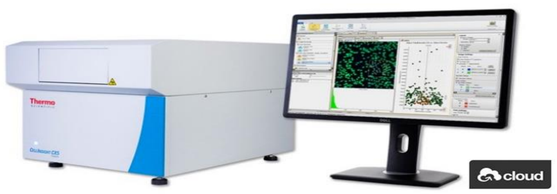
ABSTRACT

High attrition rates in drug development and lack of translation relevance can be traced back to a lack of physiological relevance when performing target identification, lead identification and optimization. Whilst more physiologically relevant, animal studies are time consuming and expensive. Therefore a niche exists for experimental models that span the gap between in vitro cell-based assays and in vivo animal studies. These systems would mimic the in vivo setting while recapitulating the ease of manipulation of a cell based assay. Traditional two dimensional cell culture models lack physiologically relevant environmental conditions. Their whole physical and biochemical setting is drastically different. For this reason researchers have been turning to three dimensional organoid and tumor spheroid systems. In this systems cells benefit from cell to cell and cell to ECM contacts. Moreover the cells exist in a more biochemically relevant state with gradients through the 3D system existing, primarily these are oxygen, nutrients and metabolites. Functionally different zones also exist within the three dimensional models with apoptotic or necrotic regions observed in one location (typically at the core) while zones of proliferative cells can be detected along the periphery. These conditions closely resemble the macro environment surrounding the cells in an intact organism. Noninvasive approaches such as fluorescence microscopy are highly advantageous as they allow for the study of these three dimensional systems. We describe the application of a suite of fluorescent biosensors in combination with automated fluorescence microscopy for the high throughput, quantitative analysis of 3D cell models. Data will be shown quantifying the induction of apoptosis in spheroid models in combination with either mitochondrial membrane potential measurements or the sequestration of therapeutic antibodies labeled with environmentally sensitive fluorescent dyes. Assessment of other pertinent cellular parameters such as viability, proliferation, cell cycle and ROS production will be demonstrated within 3D cell models. Finally, live-cell analysis of the penetration of activated immune cells into a tumor spheroid model will be shown.

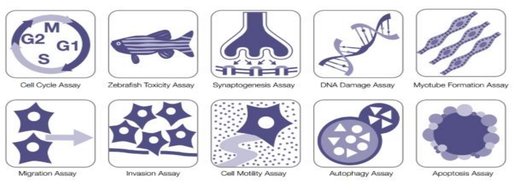
INTRODUCTION

CX7 LZR is a high content imaging instrument that now comes with laser excitation that reduces scanning times and improves axial resolution in 2D and 3D cultures. CX5 operates in five channels with LED excitation while CX7 LZR is a seven laser system with 405, 458, 488, 561, 594, 647 and 785 nm.



CellInsight CX7 LZR system highlights

Illumination	7-channel, laser-based illumination
Camera	Photometrics X1 camera
Widefield	7-channel fluorescent imaging
Brightfield	5-channel brightfield imaging
Confocal	7-channel confocal imaging
Objectives	3-position objective (range from 2x–40x, low and high NA)
Focus	Software and laser-based autofocus for consistent scan times
Automation	Configured for fully automated plate handling and scanning
Software	HCS Studio software for integrated data collection and analysis



Even more advanced performance through powerful laser illumination light source

The Thermo Scientific CellInsight CX7 LZR High Content Analysis (HCA) Platform is a fast, laser-based, automated cellular imaging and analysis platform for quantitative microscopy and phenotypic screening. It's designed to provide you with the **sensitivity and speed** that's needed for emerging assays.

RESULTS

Figure 1. Automated scanning of spheroids in bright field

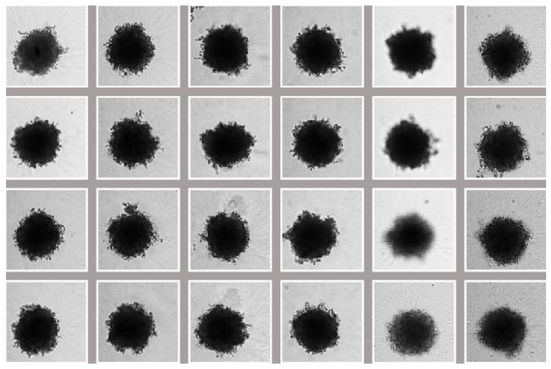


Figure 1. CX7 LZR system allows for automated imaging of spheroids. A549 cells were plated at a density of 5K/well on a Nunclon™ Sphera™ 96-well plate and incubated for 24 hrs in the CO_2 incubator. The plate was automatically imaged with 10X objective using brightfield illumination on a CellInsight CX7 LZR HCA instrument.

Figure 2. Improved axial resolution in confocal mode

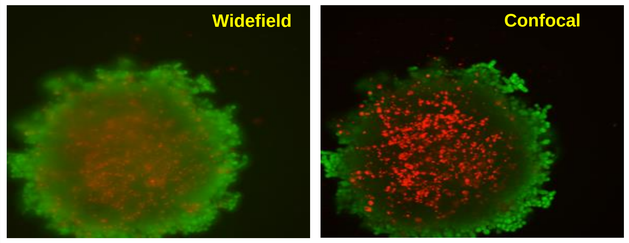


Figure 2. Confocal imaging on CX7 LZR HCA system improves axial resolution of 3D spheroid imaging. A549 cells were plated at a density of 5K/well on a corning U-bottom plate and incubated for 48 hrs in the CO_2 incubator. The plate was automatically imaged with 10X objective using wide field or confocal modes with both 561 and 488 lasers on a CellInsight CX7 LZR HCA instrument. The image is from a maximum intensity projection of 20 optical Z slices of 10 microns each.

Figure 3. Automated confocal imaging of spheroids

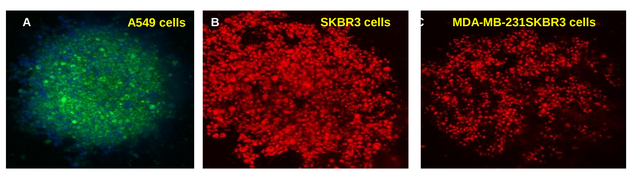


Figure 3. Confocal imaging on CX7 LZR HCA system allows measurement of hypoxic conditions and receptor internalization

Figure 3a: A549 cells were plated at a density of 5k cells/well in a 96-well Corning U-bottom plate and incubated for 48 hrs under normoxic conditions. The spheroids were then stained with 5 µM Image-iT™ Hypoxia Green probe (green) and Hoechst 33342 (blue). The plate was automatically imaged with 10X objective in confocal mode on a CellInsight CX7 LZR HCA instrument. The image is from a maximum intensity projection of 20 optical Z slices of 10 microns each.

Figure 3b and 3c: HER2+ SKBR3 or a combination of SKBR3 and HER2 – MDA-MB-231 cells (50:50) were plated in Corning U-bottom plates at a density of 10k cells/well and incubated o/n in a CO2 incubator. The spheroids were then incubated with Herceptin-pHrodo red conjugates for 48 hrs at a concentration of 1µg/ml to probe relative abundance of internalized HER2 receptors. The plate was automatically imaged with 10X objective using confocal mode on a CellInsight CX7 LZR HCA instrument using the available onstage incubator system. The image is from a maximum intensity projection of 20 optical Z slices of 10 microns each.

Figure 4: Monitoring mitochondrial health and apoptosis in spheroids

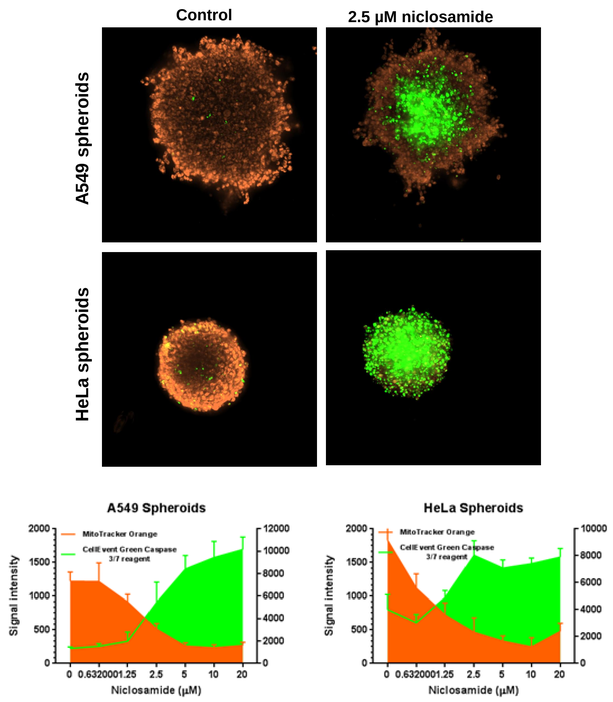
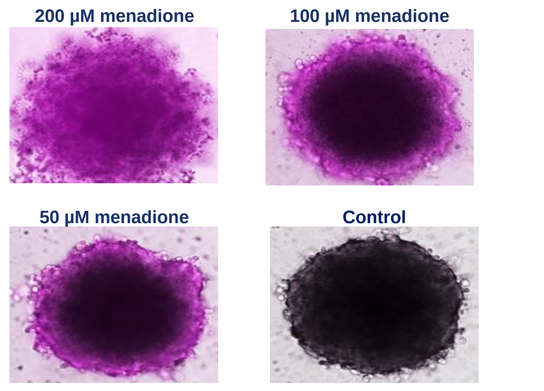


Figure 4: Mitochondrial membrane potential and apoptosis in A549 and HeLa spheroids. A549 or HeLa cells were plated at a density of 5K/well on a Nunclon™ Sphera™ 96-well plate and incubated for 24 hrs. in the CO_2 incubator. The spheroids were treated with different doses of niclosamide for 24 hrs, and then stained with 500 nM MitoTracker™ Orange and 2.5 µM CellEvent™ Green Caspase 3/7 Reagent for 1 hr. The plate was then scanned on CellInsight CX7 LZR HCA instrument. Increasing doses of niclosamide lead to depolarization of mitochondria (orange) and increase in apoptosis (green). The images are maximum intensity projections of 15 Z slices of 10 microns each.

Figure 5: Monitoring oxidative stress in spheroids



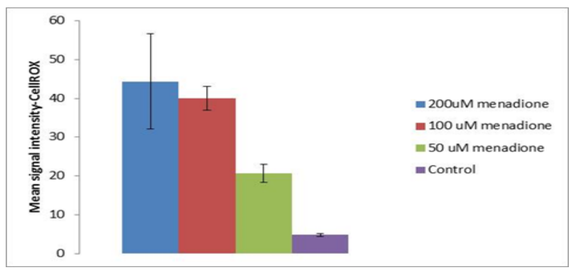


Figure 5. Endpoint measurement of ROS in spheroids. A549 cells were plated at a density of 5k cells/well in a 96-well Corning U-bottom plate and incubated for 48 hrs under normoxic conditions. The spheroids were then stained with 5 µM CellROX™ Deep Red (red). The plate was automatically imaged with 10X objective using confocal modes on a CellInsight CX7 LZR HCA instrument. The image is from a maximum intensity projection of 20 optical Z slices of 10 microns each and overlaid onto a brightfield image for display.

Figure 6. T cell killing assay in spheroids

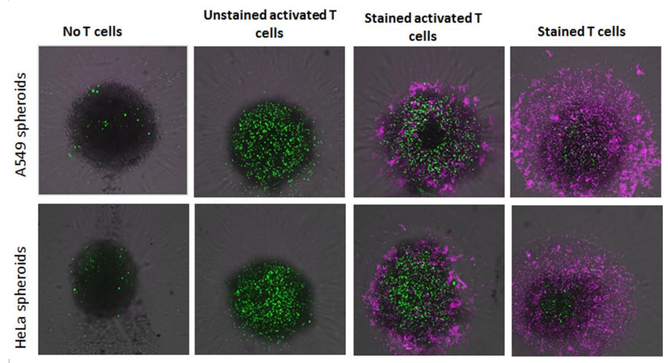


Figure 6. A549 or HeLa cells were plated at a density of 5k cells/well in a 96-well Nunclon™ Sphera™ 96-well plate and incubated for 24 hrs. Activated or normal T cells were stained with Cell Tracker Deep Red stain. About 5k T-cells were added to the spheroids and incubated for 24 hrs. the cells were then stained with 5 µM Cell Event™ Caspase 3/7 reagent for 1 hr. The plate was imaged with 10X objective on a CellInsight CX7 LZR HCA instrument using confocal mode.

Figure 7. Cell proliferation studies in spheroids

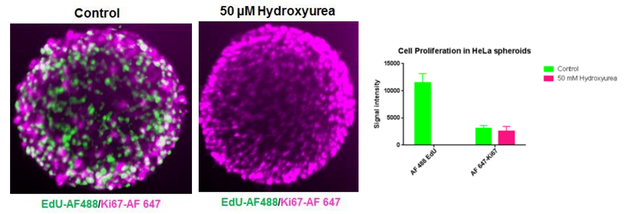


Figure 7 HeLa cells were plated at a density of 5k cells/well in a 96-well 96-well Nunclon™ Sphera™ 96-well plate and incubated for 24 hrs. The spheroids were treated with 50 µM Hydroxyurea for 24 hrs. The spheroids were then incubated with 10 µM Ethynyl deoxy uridine (EdU) for 1 hr. The spheroids were fixed and permeabilized, then washed 3x with PBS. Click reaction was done with AlexaFluor™ 488 azide for 30 mins. The cells were then stained with Ki67 antibody labelled with Alexa Fluor™ 647, using Zip Alexa Fluor™ 647 rapid labelling kit. The plate was imaged with 4X objective on a CellInsight CX7 LZR HCA instrument using confocal mode. The images are a maximum intensity projection from 15 slices of 10 microns each

CONCLUSIONS

New needs in quantitative imaging and High Content Analysis call for imaging capabilities in standard 2D models and a variety of 3D model systems, often using live cell cultures in controlled atmospheric and temperature conditions. Here, we demonstrate amenability of the CX7 LZR laser excitation system to the capture and quantification of imaging data in widefield, confocal and brightfield illumination conditions across a variety of relevant model systems, including primary neural cultures, oncogenic spheroid and standard 2D culture systems. There is also an available Onstage Incubator (OSI) module to enable chronic, dynamic measurements in live cell conditions. Live cell examples here include the use of Image-IT™ hypoxia sensor and pH sensitive pHrodo™ indicator dye systems. We also measured niclosamide induced mitochondrial membrane depolarization and apoptosis in tumor spheroids and hydroxyurea induced inhibition of cell proliferation. The confocal capabilities of the instrument also enabled the T-cell induced cell killing in spheroids. We saw significant improvements in axial resolution in 3D spheroid cultures, enabling ready quantification of signals from the surface and internal conditions with greater precision and quality than standard wide field excitation.

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