Factors to Consider for Adapting Off-the-Shelf Assays for use with 3D Culture Models

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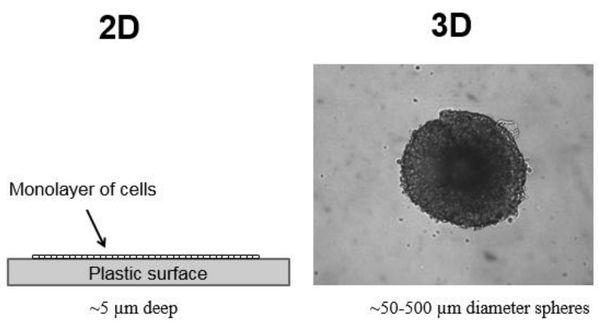
1. Introduction

Most commercially available assay kits to measure cell viability, cytotoxicity, apoptosis, reporter gene activity, etc. were designed using monolayer cultures that may be only ~5µm thick. However, 3D cell culture models have much larger physical dimensions that may require 100s of µm of reagent penetration. There is an unmet need for assays that have been validated to measure markers in larger 3D culture models. Two basic factors to consider include: 1) the ability of the detection reagent to lyse all the cells in large 3D structures and 2) the ability of small molecule detection probes to penetrate through the entire 3D structure. We will present examples of changing the reagent formulation and/or the assay protocol to improve the ability to lyse cells. We will also describe adapting the use of cell permeable and nonpermeable small molecule probes to detect viable and dead cells in real time assays. Although each cell type and 3D model system can be expected to have different characteristics, we will make general recommendations as a starting point for factors to consider to validate assays for measuring cell health parameters in 3D culture models.

2. What is Different Between 2D and 3D Cultures?

The thickness of the cell layers affect the ability of assay reagents to penetrate and lyse cells.

The mass of cells may also affect imaging to the center of structures.



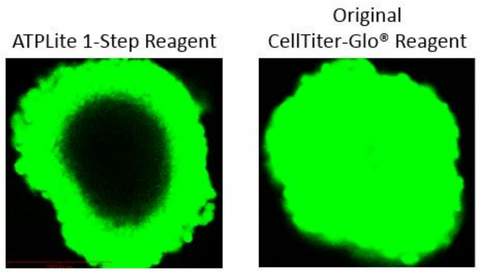
3. Not All Lytic Assay Reagents are Equal

Critical experiment prompting efforts to validate assays for 3D

"Off-the-shelf" reagents may not be validated for use with 3D cell culture model systems.

A comparison of the original CellTiter-Glo® ATP Assay and the ATPLite 1-Step reagent showed a big difference in the ability to lyse all cells in ~350µm diameter spheroids.

Large 3D spheroids may require special treatment to ensure complete lysis and extraction of the marker being measured.



HCT116 cells were cultured in InSphero GravityPLUS™ 3D Cell Culture system to form ~350 mm microtissues. The CellTox™ Green DNA binding dye was added to the ATP detection reagent and samples processed according to manufacturer's protocols, then photographed using confocal microscopy.

4. Reagent Reformulation to Improve Cell Lysis

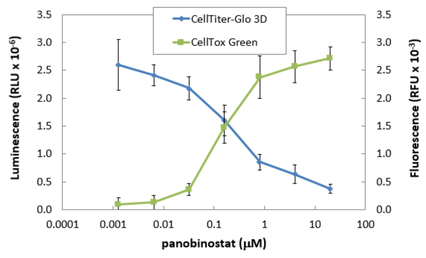
CellTiter-Glo® 3D Cell Viability Assay / Optimized for 3D

The original CellTiter-Glo® Luminescent Cell Viability Assay was reformulated by changing the detergent ingredients to improve lysis of cells in large 3D culture models.

Reformulation to create a robust reagent was enabled by use of the heat and detergent resistant Ultra-Glo™ Recombinant Luciferase.

CellTiter-Glo® 3D has been widely adopted to estimate viable cell number in 3D cell models and can be multiplexed with other methods.

Multiplexing ATP Detection & DNA Staining using 3D Spheroids



HCT116 cells were cultured in InSphero GravityPLUS™ 3D Cell Culture system for 4 days to form ~350 mm microtissues. Samples were treated with CellTox™ Green and panobinostat for 48 hr. After recording fluorescence, an equal volume of CellTiter-Glo® 3D Reagent was added, plate was shaken for 5', and the luminescence was recorded after a 30' incubation.

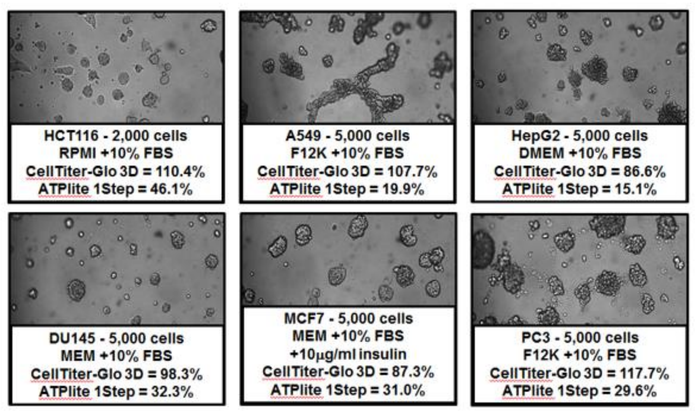
5. Appropriate Controls to Establish Percent Recovery of Extracted Marker

Acid extraction is the gold standard for extracting ATP.

Acid treatment of samples followed by neutralization and measurement (compared to an ATP standard curve) is used to establish % recovery of ATP.

The % recovery of ATP from 6 different cell types cultured with Matrigel was compared between the CellTiter-Glo® 3D and ATPLite assays.

Cell types forming larger 3D aggregates show higher recovery of ATP using the CellTiter-Glo® 3D Assay.



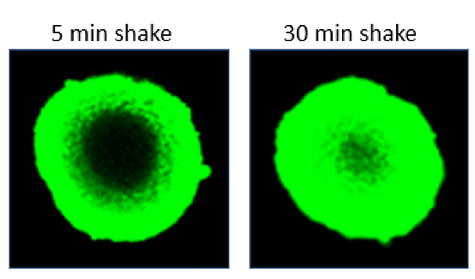
6. Protocol Modification to Achieve Cell Lysis

Caspase-Glo® 3/7 Assay protocol modification

If reformulation of reagent to strengthen detergent is not possible, modification of the assay protocol may accomplish effective extraction of the desired marker from 3D structure.

Caspase activity is not stable to high concentrations of detergent.

Modifying the protocol to lengthen mixing time to 5 minutes and incubate with lysis solution for a total of 30 minutes resulted in near complete lysis and improved recovery of caspase 3/7 activity from large spheroids.

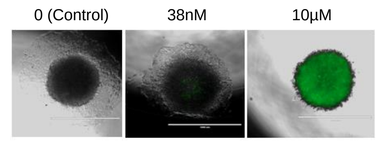


HCT116 cells were cultured in InSphero GravityPLUS™ 3D Cell Culture system to form ~330 mm microtissues. The CellTox™ Green DNA binding dye and Caspase-Glo® 3/7 Reagent were added and samples shaken for 5 or 30 minutes before photographing using confocal microscopy.

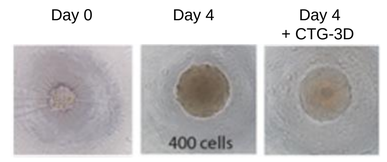
7. Microscopic Observation of Spheroids May be Misleading

Microscope images may suggest spheroids are relatively intact even if the cell membranes have been lysed to release markers such as ATP or caspase activity.

Spheroid structure appears relatively intact even after treatment with panobinostat and stained with CellTox™ Green DNA binding dye indicating loss of membrane integrity (Panel A, right image) or treated with CellTiter-Glo® 3D Assay reagent to lyse cells (Panel B, right image).



Panel A. HCT116 cell spheroids formed in ultra low binding plates and treated with panobinostat were subsequently stained with CellTox™ Green DNA binding dye (to indicate dead cells) and photographed using light or fluorescent microscopy. Note spheroid in right image appears intact even though all cells have been lysed. Bar = 1000µM



Panel B. HCT116 cell s added to ULA plates and photographed on Day 0 (left) and Day 4 (center). CellTiter-Glo® 3D Reagent was added, the plates shook for two minutes and photographed.

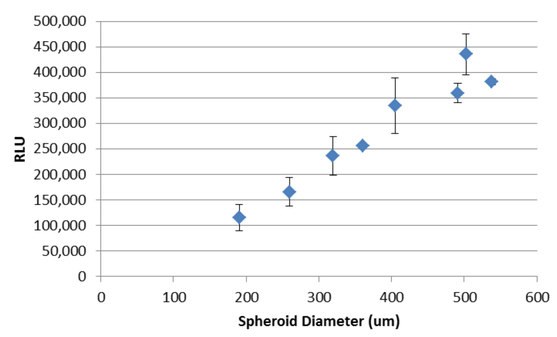
8. Cell Permeable Reagents Do Not Require Cell Lysis

RealTime-Glo™ Assay used with 3D Spheroids

One option to avoid the difficulty in lysing all the cells in 3D structures is to utilize assay reagents that are cell permeable.

The RealTime-Glo™ MT Cell Viability Assay contains a cell permeable "pro-substrate" for NanoLuc® Luciferase.

The RealTime-Glo™ Assay may be a suitable approach for estimating viable cell number in 3D models.



Spheroids of varying sizes were generated by adding different numbers of cells to the InSphero GravityPLUS™ 3D Cell Culture system. Luminescence from the RealTime-Glo™ Assay correlates with diameter of spheroid.

9. Summary / Take Home Message

- Most *in vitro* cell-based assays were originally designed for 2D monolayers
- Off-the-shelf assays should be validated to ensure they work with 3D models
- Size of 3D structures may limit efficient lysis of all cells
- Reformulation of lysis solution may improve extraction of marker from 3D cell structures
- Optimizing the mixing and incubation time with lysis solution may improve cell lysis if reformulation of detergent is not possible
- Non-lytic "real time" viability and apoptosis assay options are available for 3D culture models

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