Design and Validation of Bioluminescent Assays for 3D Cell Culture Models

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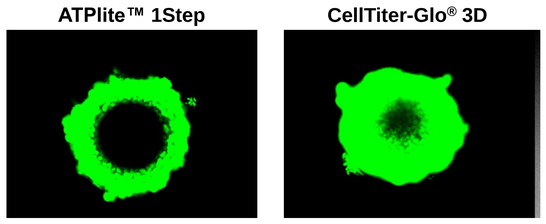
Abstract #226

1. Introduction

Cells cultured in 3D model systems often acquire relatively large in vivo-like structures compared to the thickness of a 2D monolayer of cells grown on standard plastic plates. Multicellular 3D culture systems containing more than one cell type and exhibiting formation of a complex extracellular matrix represent a more physiologically relevant environment, yet provide a challenge for assay chemistries originally designed for measuring events from monolayers of cells. There is an unmet need for guidelines for design and verification of convenient and effective assays useful for larger 3D microtissues. Critical factors to consider for each model system include effective penetration of detection reagents and/or complete lysis of microtissue structures using combinations of detergent and physical disruption. We have developed an improved reagent formulation for bioluminescent detection of ATP for measuring cell viability. The improved formulation demonstrates more effective lysis of large microtissues. Results from modifying assay procedures to include more rigorous physical disruption of microtissues will be presented for measuring caspase activity for detecting apoptosis and cell stress assays to detect mechanisms leading to cytotoxicity.

2. New ATP assay formulation shows improved lytic capacity

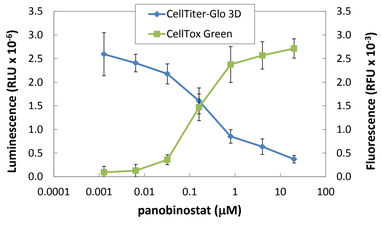
The new CellTiter-Glo® 3D Reagent shows improved cell lysis of 3D microtissues compared to ATPlite™ 1Step.



HCT116 cells were cultured in InSphero GravityPLUS™ 3D Cell Culture system for 4 days to produce ~ 300 µm diameter spheroids. CellTox™ Green (DNA-binding dye not permeable to live cells but staining dead cells) was added to each ATP assay reagent prior to adding to spheroids.

3. Multiplexing ATP assay and DNA staining

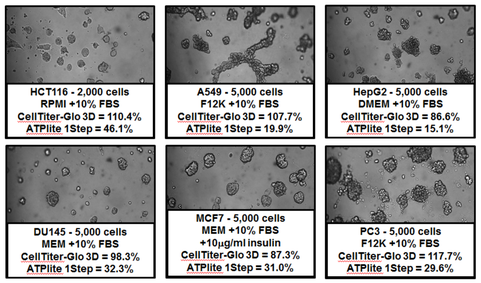
CellTox™ Green is a membrane impermeable DNA-binding dye that selectively stains dead cells. CellTox™ Green can be multiplexed with CellTiter-Glo® 3D ATP Assay.



HCT116 cells were cultured in InSphero GravityPLUS™ 3D Cell Culture system for 4 days to form ~350 µm 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, the plate was shaken for 5', and the luminescence was recorded after a 30' incubation.

4. Detection of ATP from Matrigel® cultures

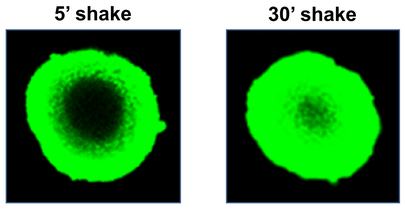
Comparison of ATP assay formulations for recovery of ATP from various cell types in Matrigel ®.



Cells were cultured in Matrigel® coated plates for 4 days, then assayed with optimized protocol for each reagent. % recovery was based on comparison to TCA extraction method.

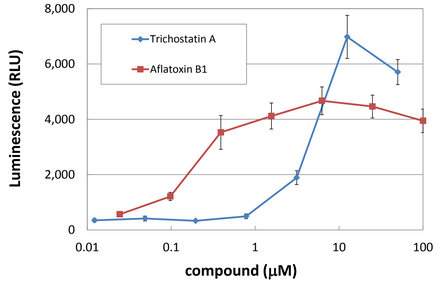
5. Caspase assay protocol for microtissues

A modified protocol for Caspase-Glo® 3/7 shows a longer shake time more effectively lyses cells in large microtissues.



CellTox™ Green was combined with Caspase-Glo® 3/7 reagent prior to adding to ~330 µm HCT116 microtissues. Samples were shaken for either 5 min (then incubated for 25 min) or shaken 30 min prior to imaging using confocal laser fluorescent microscopy.

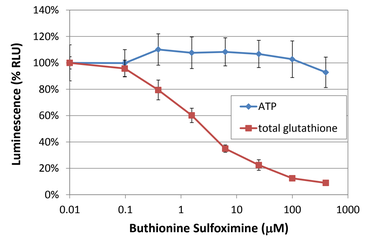
6. Caspase assays of microtissues



Human liver microtissues (~275 um) were treated with Trichostatin A or Aflatoxin B1 for 48 hr. An equal volume of Caspase-Glo® 3/7 was added to the samples, they were shaken for 30 min prior to recording luminescence.

7. Glutathione assay of microtissues

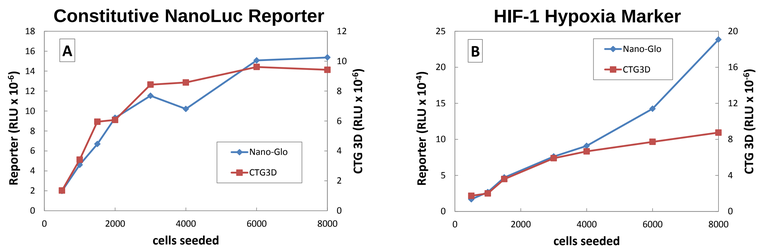
Cell viability and oxidative stress can be determined by assaying parallel samples with CellTiter-Glo® 3D and GSH/GSSG-Glo™.



For GSH assay, the medium was removed from 4-day ~350 µm HCT116 spheroids treated for 48 hr with buthionine sulfoximine. 50µl of total glutathione lysis reagent was added and the plates shaken for 30'. 50µl of luciferin generation reagent was then added, and after a 30' incubation, 100 µl of luciferin detection reagent was added. After a final 15' incubation, luminescence was recorded. ATP was assayed from parallel samples using the CellTiter-Glo® 3D Assay.

8. Measurement of luciferase reporter activity from different size microtissues

HIF-1 promoter driven hypoxia marker expression increases in larger microtissues.



HCT116 cells expressing NanoLuc luciferase under a constitutive promoter (A) or a HIF-1 promoter (B) were cultured in InSphero GravityPLUS™ 3D Cell Culture system for 4 days to form ~200-700 µm microtissues. An equal volume of NanoGlo Reagent or CellTiter-Glo® 3D Reagent was added to each well, the plate shaken for 10', and luminescence recorded after a total of 30 min incubation.

9. Summary

A number of luminescent assays have been successfully applied to 3D microtissues for detection of:

- **ATP for cell viability (CellTiter-Glo® 3D)**
- **DNA staining of dead cells (CellTox™ Green)**
- **Caspase marker of apoptosis (Caspase-Glo® 3/7)**
- **Glutathione as marker of oxidative stress (GSH/GSSG-Glo™)**
- **Luciferase reporter expression (Steady-Glo®, ONE-Glo™, Nano-Glo®, etc.)**

Current efforts are aimed at further validating these assays, and others (e.g. ROS-Glo™) using hanging drops, hydrogels and synthetic scaffolds (e.g. Alvetex).

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