In vitro 3D Spheroids and Microtissues: ATP-based Cell Viability and Toxicity Assays

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Abstract

In vitro models continuously evolve to more closely mimic and predict biological responses of living organisms. Just in the past years many novel three dimensional (3D) organotypic models, which resemble tissue structure, function and even disease progression, have been developed. However, application of more complex models and technologies may increase the risk of compromising assay robustness and reproducibility. Consequently, the first developmental stage of cell-based assays is to combine complex tissue models with standard assays - a combination that already provides more physiologically insightful information when compared to two-dimensional (2D) systems. The final goal should be to exploit the full potential of tissue-like in vitro models by investigating them with modern assays such as −Omics and imaging technologies. Furthermore, organotypic models will allow for a design of novel assay concepts that utilize the whole tool box of models and endpoints.

In this chapter we focus on assessment of spheroid viability by measuring intracellular ATP content. This primary assay performed on 3D cell culture system is a powerful tool to predict with high confidence health, growth and energy status of tissue of interest. The 3D spheroid model is particularly useful to mimic solid-tumors from a physiologically relevant architectural perspective, when they are grown with multiple cell types prevalent in these tumors. However, this assay is equally applicable for other non-spheroid 3D tissue models to quantify viability and toxicity.
Introduction

In recent decades cell-based assays to investigate cell biology, drug efficacy, metabolism and toxicology were dominated by technologies employing cells grown on flat plastic surfaces (2D) or in single cell suspension (1). However, biology of cells is extensively influenced by the environmental context such as cell-cell contacts, cell-matrix interactions, cell polarity or oxygen profiles.

For many years biology of avascular tumor has been recognized by cancer researchers to be particularly well mimicked by three dimensional (3D) cell cultures (2)(3). For instance, one of the earliest 3D-acknowledged effects correlating with in vivo clinical observations was development of multicellular resistance (MCR) to anticancer drugs in 3D culture formats. As highlighted by Desoize and Jardillier, cancer cells embedded within a 3D environment had lower sensitivity to anticancer drugs, e.g. upon Vinblastine exposure human lung carcinoma (A549) monolayer culture exhibited the IC$_{50}$ value of 0.008 µmol/l, whereas the IC$_{50}$ value of spheroid culture was 53 µmol/l (4). Importantly, drug sensitivity is a net effect of multiple factors and is highly regulated by hypoxia, which occurs in the oxygen-deficient areas of the tumor with limited access to the capillary network. Low oxygen partial pressure can lead to either higher drug sensitivity or elevated drug resistance of the tumor, depending on the drug mechanism and structure. Additionally, extracellular acidification is yet another factor influencing response to either basic (e.g. Doxorubicin) or acidic (e.g. Chlorambucil) drugs. In this case, the uptake of basic drugs is decreased, whereas the uptake of acidic drugs is increased, resulting in higher drug resistance or higher drug sensitivity, respectively (5) (6). Therefore, tumor cells cultured in 3D formats, which are exposed to complex and heterogenic environmental context, are more relevant tool to study tumor biology and responsiveness than standard 2D cell culture.
Another example of cells with well documented influence of culturing conditions on physiology are hepatocytes. Hepatocytes are characterized by their polygonal shape and multi-polarization with at least two basolateral and two apical surfaces. Changes in cell form limit cell–cell and cell–matrix interactions, consequently leading to reduced polarization, reduced bile canaliculi formation and a loss of important signaling pathways. Dedifferentiation of hepatocytes observed in 2D monolayer cell culture results in reduction of liver-specific functions, such as metabolic competence for detoxification, due to down-regulation of phase I and II enzymes. Therefore, maintenance of hepatocyte shape and function is of the utmost importance in hepatotoxicity studies. To tackle this problem, 3D liver models employing scaffolds, hydrogels and the cellular self-assembly approach have been created. Additionally, variety of different cell types, such as HepG2, HepaRG and primary hepatocytes, is currently used to investigate liver functions. For an in depth overview of current in vitro liver models and application please see Godoy et al. 2013 (7).

However, a decision about application of a cell-based methodology depends not only on its physiological properties, but also on its automation-compatibility, high throughput processing and feasibility to couple it with established endpoint. A number of technologies have been developed to create 3D tissue-mimicking environment on microscale in vitro, with embedding cells within a hydrogel or preventing of cellular adhesion to an artificial matrix and concomitant enforced cell re-aggregation being the main ones (Table 1) (8)(9) (10)(11)(12). Both scaffold-free technologies have been used successfully to create a 3D context of cancer cells, as they allow for reconstitution of cell type-specific extracellular environment. The concept of the hanging drop technology is one of the oldest ones and it provides the benefit of aggregation of defined types and number of cells. Already used by Ross Granville Harrison, the hanging drop has proven to be a universal technology to produce a wide variety of either disease models or primary tissues (7)(13)(14)(15)(16).

A paradigm of 3D spheroid/microtissue growth and assay development summarized in Flow Chart 1, shows interplay between selection of the most suitable cell type(s) and the 3D culturing technique, followed by optimization of spheroid culturing conditions and morphology, and between the assay development and the choice of the endpoint. Tailored combinations of the above elements offer experimental freedom that makes the 3D in vitro testing systems fit to the purpose, and increase the amount of extracted biologically-relevant information.

1 In Vitro Toxicity and Drug Efficacy Testing in a 3D Spheroid Model

1.1 In Vitro Toxicity and Drug Efficacy Assay Concept

An increasing need for robust and reliable in vitro models for toxicity and drug efficacy testing is potentiated not only by the urge to make the process of bring therapeutics from the bench to the bedside faster and more cost-effective, but also by increasing regulatory and safety challenges. However, one of the major concerns of in vitro toxicity/efficacy testing remains its predictive power and translation into in vivo situations. As discussed in the introductory section, 3D cell culture formats such as spheroids, present a powerful alternative to standard 2D cell culture for in vitro studies (17)(18). The 3D spheroid model is particularly useful to mimic solid-tumors from a physiologically relevant architectural perspective, when they are grown with multiple cell types prevalent in these tumors.

The choice of the 3D model and the end point for toxicity/efficacy testing should depend on both the physiological question to be answered and the scale of the screen. In general, treatment with a toxicant can affect cellular and/or 3D cell culture morphology, viability, metabolic activity (such as oxygen consumption or metabolic enzyme activation), or tissue-specific function. Here, the spectrum of possible tissue-specific end points is constantly
widening, together with the development of specialized 3D tissue models (19)(20)(21)(22)(23)(24)(25). In case of liver microtissues of co-cultured hepatocytes and non-parenchymal cells (NPCs), established approaches include monitoring albumin and urea secretion, bile acid secretion, Kupffer cell-dependent IL-6 and TNFα secretion, to list a few (26). Contractile responsiveness of the myocardial microtissue model or glucose-stimulated insulin secretion by pancreatic microislets add yet further options to the growing list of functionality tests. Additionally, cultivated 3D cell culture models can be further analyzed using transcriptomic and proteomic methods, allowing for RNA and protein expression profiling upon toxicant exposure.

Although very powerful and promising, the use and predictivity of the 3D models has to be carefully validated for each given application, and conditions of cultivation and sample collection need to be standardized and controlled (27)(28). For screening purposes 3D cell culture models can be treated with many classes of substances (e.g. small molecules, biologicals, siRNA/RNAi). It is good practice to include an appropriate model- or cellular process-specific control compound of known toxic effect, such as Chlorpromazine for drug-induced hepatotoxicity, Aflatoxin B for apoptotic cell death induction or Trovafloxacin for inflammation-mediated toxicity (26).

In this section we will describe an exemplary experimental design to test the toxic effects over a range of concentrations of compounds dissolved in tissue culture-grade dimethyl sulfoxide (DMSO, 0.5% v/v) on liver microtissues of primary hepatocytes co-cultured with primary NPCs, produced in a hanging drop technology (Figure 1) (26). Analogously, such an experimental set up can be applied to evaluate anticancer efficacy of drugs in spheroids derived from cancer cell lines, such as HEY - human ovarian cancer cell line, as presented in Figure 2. The effect of the toxic agents on microtissue morphology, cell viability and tissue functionality can be further investigated, depending on the study goal, endpoint of interest and compatibility with the screening approach.

### 1.1.1 Sample Protocol for a Commercially Available 3D Spheroid System

The GravityPLUS™ Hanging Drop System is designed to generate organotypic microtissues in the process of scaffold-free aggregation of cells and to enable for their prolonged cultivation and multiple compound re-dosing. Microtissues are formed within 2-4 days from cell suspensions in hanging drops on the GravityPLUS™ Plates and are subsequently harvested into the ultra-low adhesive GravityTRAP™ ULA Plates. The unique design of GravityTRAP™ ULA wells allows for numerous media exchange without microtissue disturbance as well as for microtissue imaging. This 96-well platform is compatible with liquid handling stations and suitable for HT-screening applications. Commercially available hanging drop system and microtissues for hepatotoxicity testing include:

- GravityPLUS™ 10x Kit (96-well), includes 10 GravityPLUS™ and 10 GravityTRAP™ ULA plates (InSphero, Cat.# CS-06-001)
- GravityTRAP™ ULA Plate (InSphero, Cat.# CS-09-001)
- 3D InSight™ Human Liver Microtissues from primary hepatocytes, co-culture with non-parenchymal cells (96x) (InSphero, Cat.# MT-02-002-04)
- 3D InSight™ Human Liver Microtissues from primary hepatocytes (96x) (InSphero, Cat.# MT-02-002-01)
- 3D InSight™ Rat liver microtissues formed by primary hepatocytes (96x) (InSphero, Cat.# MT-02-001-01)
- 3D InSight™ Rat liver microtissues from primary hepatocytes, co-culture with nonparenchymal cells (96x) (InSphero, Cat.# MT-02-001-04)
1.1.2 Compound Preparation

1. To adjust 0.5% DMSO (v/v) final concentration in culture medium, prepare a 200 X top compound concentration stock in DMSO.
2. Prepare 6 dilutions of the compound stock in DMSO using sterile V-bottom microplate (e.g. Greiner Bio-one, Cat.# 651161). Choose the dilution factor depending on the range of concentrations to be tested in the assay.
3. Transfer 2.5 µl of each compound dilution to the corresponding well on a deep well plate (e.g. Axygen®, Cat.# 391-01-111) as presented in Figure 3 (upper panel). For each re-dosing prepare a separate deep well plate.
4. For vehicle control, pipette 2.5 µl of DMSO to columns 3 and 4 on a deep well plate.
5. Seal deep well plates with aluminum plate sealer (e.g. Greiner Bio-One, Cat.# 67609) and store in –20°C for future re-dosing.

1.1.3 Compound Exposure Protocol

1. Thaw deep well plates with compound(s) to be tested and add to each experimental well 497.5 µl of pre-warmed culture medium, thereby generating 1 X top concentration of the compound and its corresponding dilutions in culture medium with 0.5% DMSO (v/v).
2. Gently aspirate culture medium from the GravityTRAP™ ULA Plate, leaving microtissues in the remnant volume of the medium in the V-shaped bottom of the well.
3. Thoroughly mix medium with compound in the deep well plate and dose 70 µl per microtissue in required number of replicates (Figure 3, middle panel).
4. To control the DMSO effect on microtissues, add 70 µl of culture medium per well to column 2 on the Gravity GravityTRAP™ ULA Plate (Figure 1, middle panel).
5. Repeat dosing at required time intervals.
6. Determine toxicity and cell viability using CellTiter-Glo® 3D Cell Viability Assay or other suitable methods available.

1.2 Conclusion/Summary

Testing toxicity/drug efficacy in 3D cell culture formats presents multiple advantages over conventional 2D cell culture system. Firstly, cells aggregated into a 3D structure exhibit native tissue-mimicking organization, metabolic characteristics and specialized functions, and retain them for significantly longer periods of time, therefore enabling prolonged and repeated exposure. This in turn allows for detection of effects caused by longer exposure of lower compound concentrations, which appears frequently in vivo. For example, longer exposure tends to shift IC_{50} values towards lower compound concentrations, hence increasing sensitivity of the assay and better prediction of false negative compounds (Figure 1). Additionally, a comparison between shorter and longer toxic exposures may give an idea about sensitization of the system to a given treatment. Secondly, several commercially available solutions allow for 3D cell culture cultivation in HT-friendly 96- or 384-well format with multiple re-dosing of tested compounds. On the assay development side, an appealing concept of multiplexing endpoints to generate simultaneous data-reach readouts is currently under development and shall provide more experimental flexibility.
2 3D Microtissue Viability Assay

2.1 ATP Assay Concept

The frequently chosen primary assay for determination of 3D cell culture viability is quantification of a luminescent signal generated by conversion of luciferin by luciferase as a function of cytoplasmic ATP concentration. Initially, architecture of the 3D cellular aggregates – their size, composition and penetration barrier, presented a challenge to assays originally tailored for the 2D cell culture models. However, by optimization of detergent composition and lysis conditions, ATP assays suitable for variable 3D cell culture formats (such as spheroids and hydrogel-based systems), have been developed. Available bioluminescent ATP detection assay are robust, sensitive, and scalable to high-throughput screens, and offer relatively simple work-flow and data analysis. In contrast, standard colorimetric methods based on resazurin reduction (Alamar blue assay) or tetrazolium reduction (MTT assay), frequently used to assess number of viable cells in 2D cell culture, have been found not applicable to 3D spheroids/microtissues and collagen matrices. 3D matrices and tight cell-cell junctions can affect uptake and diffusion kinetics of a dye, therefore changing readout of the assay and making results more difficult to interpret. In parallel, development of live imagining assays linking changes of spheroid’s size and morphology or localization/expression of fluorescent markers to viability of cells in 3D formats are under constant development.

The protocol below describes how to measure viability of cells aggregated into spheroids (e.g. heterotypic liver microtissues and tumor microtissues) using CellTiter-Glo® 3D Cell Viability Assay quantifying intra-tissue ATP content (Figure 1 and Figure 2). This protocol can be easily adjusted to an automated pipetting station.

2.1.1 Commercial Availability

Recommended single-reagent assay for multi-well plate format:

- CellTiter-Glo® 3D Cell Viability Assay, Promega Corporation, Cat.# G9681, G9682, G9683

CellTiter-Glo® 3D Cell Viability Assay combines the enhanced penetration and lytic activity required for efficient lysis of 3D cell culture with generation of the stable ATP-dependent luminescent signal. This thereby reduces the complexity of processing multiple assay plates and HTS applications.

2.1.2 ATP Assay Preparation

CellTiter-Glo® 3D Cell Viability Assay is provided as a ready-to-use solution and no additional preparation is required. The reagent should be equilibrated to room temperature before use. For stability and storage conditions please refer to the manufacturer’s guidelines (www.promega.com).

To perform the assay on microtissues cultured in 96-well GravityTRAP™ ULA Plates, mix 1:1 the required volume of CellTiter-Glo® 3D Cell Viability Assay (20 µl per well) and PBS without calcium and magnesium (e.g. PAN-Biotech, Cat.# P04-36500).

2.1.3 ATP Assay Protocol

1. Equilibrate GravityTRAP™ ULA Plates with cultured micro-tissues to room temperature.

2. Prepare 96-opaque well microplate, hereinafter refer to as assay plate (e.g. Greiner Bio-One, Cat.# 675075), by pipetting into dedicated wells (Figure 3, lower panel):
a Blank – 40 µl of diluted CellTiter-Glo® 3D Cell Viability Assay

b Optional: Standard curve – depending on the type of microtissue and the detection range of luminometer available, mix 20 µl of CellTiter-Glo® 3D Cell Viability Assay with 20 µl of 1 µM ATP (e.g. for human liver microtissues of ~ 300 µm diameter) or with 5 µM ATP (e.g. for more metabolically active or bigger microtissues), and with corresponding ATP dilutions.

c Optional: To check background interference of the compound tested in the cytotoxicity assay, pipet 5 µl of a culture medium from wells containing microtissues treated with the highest concentration of the compound into wells on the assay plate containing 20 µl CellTiter-Glo® 3D Cell Viability Assay and 20 µl of 1 µM ATP.

3 Gently remove the culture medium from the GravityTRAP™ ULA Plate by placing the pipette tip at an inner ledge of the well, leaving intact the microtissues in the remnant volume of the medium in the V-shaped bottom of the well.

4 Dispense 40 µl of diluted CellTiter-Glo® 3D Cell Viability Assay into each well of the GravityTRAP™ ULA Plate.

5 Mix and transfer content of each well from the GravityTRAP™ ULA Plate into the corresponding well on the assay plate.

6 Protect the lysate from light by covering the assay plate with aluminum foil or with aluminum platesealer (e.g. Greiner Bio-One, Cat.# 67609).

7 For effective MT lysis keep the plates on an orbital shaker for 20 min at room temperature.

8 Record luminescence with a microplate luminometer using a program recommended by the manufacturer.

2.1.4 Data analysis

The absolute ATP concentration of microtissue can be calculated from the standard curve included on the same assay plate. However, for in vitro testing of cell toxicity of chemicals, it is often more applicable to calculate relative ATP levels of microtissues exposed to treatment as a percentage of vehicle-treated control microtissues (Figure 1, Figure 2). During prolonged cultivation of microtissues, certain cytotoxicity and a decrease in ATP levels of DMSO controls can be observed with respect to maintenance medium controls.

2.2 Conclusions/Summary

The type and number of cells integrated into the 3D structure as well as cultivation conditions (cell culture media compositions, time of cultivation, media exchange/re-dosing scheme) may affect physiological characteristics of the model and its responsiveness to the treatment. Therefore, standardization of intrinsic characteristics of 3D cell culture formats and extrinsic culturing parameters and protocols is crucial for further development of 3D in vitro assay portfolio.

Measurement of cytoplasmic ATP content is a common method for cellular viability determination in both 2D and 3D cell culture, and is a routine endpoint in toxicology/drug efficacy studies. However, 3D culture formats are characterized by development of compact structures with tight cell-cell junctions and extracellular matrix, presenting additional obstacle for effective lysis and reagent accessibility. Therefore, to allow for effective ATP release from cells, the time of lysis combined with physical disruption of the 3D structure should be determined empirically for each 3D cell culture format.
Ready-to-use assay kits, such as CellTiter-Glo® Cell Viability Assay, facilitate time-effective and standardized processing of multiple assay plates by combining lysis and luminescent signal generation into one step. However, for the best assay performance special care should be taken to ensure both the highest system reproducibility and operational reproducibility (e.g. mixing and transfer of the reagent with 3D culture from culturing plates to the assay plates, avoiding a temperature gradient within the plate and ATP contamination). Additionally, special care should be taken to ensure that the ATP levels of either large or metabolically active 3D cultures correlate with the dynamic range of luminescence output of the assay.

References

14. Kelm JM, Timmins NE, Brown CJ, Fussenegger M, Nielsen LK. Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. Biotechnol...


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Table 1:
Overview of the three basic cell culture concepts that are employed to coax cells into a 3D environment.

<table>
<thead>
<tr>
<th>Concept</th>
<th>Characteristics</th>
<th>Examples</th>
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<tr>
<td>Scaffold-free</td>
<td>Cells produce their own ECM, no scaffold-associated growth factors/undefined components, no scaffold-associated batch-to-batch variations, possibility to co-culture different cell types, cellular aggregation in hanging drops or in ultra-low attachment plates, HTS-applicable.</td>
<td>Multicellular tumor spheroids (14)(39), Colorectal cancer spheroids (40), Human liver heterotypic microtissues (26), Cerebral organoids (41), Myocardial microtissues (42)(23), Neurospheres (43), Kidney organoids (44).</td>
</tr>
<tr>
<td>Hydrogels/sponges/membranes</td>
<td>Mimic soft tissue stiffness and ECM composition, natural tissue-derived (batch-to-batch variations, biological contaminants, e.g., growth factors) or synthetic, gel maintenance conditions may limit application.</td>
<td>Mini-guts (45).</td>
</tr>
<tr>
<td>Scaffolds/matrices</td>
<td>Added (biodegradable) cellular, acellular or composite materials, mimic ECM, diversified pore geometry, mechanistic properties, size and transparency, regenerative medicine/implants.</td>
<td>Pre-vascularized scaffolds for bone regeneration (46).</td>
</tr>
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Figure 1:
Toxicity testing in 3D heterotypic human liver microtissues - reproducibility and sensitivity of the ATP assay. (A) The human liver microtissues (hLiMTs) of co-cultured primary hepatocytes and primary NPCs were cultured for 14 days and their intratissue ATP content was assessed with Promega CellTiter-Glo® assay. In each assay (n = 40) average relative light units (RLU) from triplicates (3 microtissues) was set to 100%, the relative standard deviation (SD) of the mean is depicted. Average relative SD from 40 assays is 14.6%. (B) Reproducibility of IC₅₀ values of Chlorpromazine after 7 days- and 14 days-long treatment of hLiMTs. Presented are results of independent experiments and their geometric mean. Note the reproducible shift to lower IC₅₀ values after increased exposure time. (C) hLiMTs were exposed to increasing concentrations of Tolcapone and of Diclofenac (D) during shorter (5 days and 7 days, respectively; 1 re-dosing) or longer (14 days; 2 re-dosing) incubation. Note the shift to lower IC₅₀ values after increased exposure time. Source: InSphero AG.
Figure 2:
Anticancer drug efficacy testing in tumor microtissues - correlation between cell viability and tumor microtissue size suppression. Tumor microtissues grown from human ovarian cell line (HEY) were exposed to increasing concentrations of Doxorubicin (A), Staurosporine (B) and Cisplatin (C) for 10 days and their intra-tissue ATP content was assessed with Promega CellTiter-Glo® assay. Representative images of control and compound-treated microtissues show dose-dependent decrease of microtissue size upon treatment with Doxorubicin (A) and Staurosporine (B) which corresponds to decrease of microtissue viability as measured with the ATP assay. Cisplatin treatment (C) neither surpressed microtissue viability nor had an impact on size of spheroids. Source: InSphero AG.

Figure 3:
Schematic plate layout of compound-treated spheroids and of ATP measurement. Upper panel: compound deep-well plate layout. Each row contains vehicle control (column 3 and 4) and 7 compound concentrations (column 5 – 11; top concentration: column 11). Application of deep well plates reduces pipetting steps to generate 200 X dilutions of the compound and allows for dosing of experimental replicates from the same reservoir. Middle panel: dosing of microtissues in 96-well format. Microtissues cultured in GravityTRAP™ ULA Plate are exposed to treatment with 2 compounds. Each compound concentration is tested in quadruplicate, whereas the vehicle control in octuplicate. Culture medium control is included in column 2. Lower panel: assay plate layout for ATP measurement. Microtissues suspended in 40 µl of diluted CellTiter-Glo® Cell
Viability Assay are transferred from the GravityTRAP™ Plate into the assay plate (column 2 to 11). Assay blank (A1), standard curve (A1 – D1) and the control for background interference (column 12) are included in the assay plate.