Progressing 3D Spheroid Analysis into a HTS Drug Discovery Method

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Introduction

Inhibition of cancer cell proliferation in drug discovery research has not translated well from traditional two-dimensional (2D) *in vitro* assays to *in vivo* studies. Most anti-cancer drug compound studies are performed in a tissue culture treated 2D assay format for the purpose of studying proliferation, viability, and apoptosis. Increasingly, scientific evidence is showing that growing cancer cells in the form of three-dimensional (3D) spheroids is more predictive of *in vivo* study outcomes than 2D cell culture formats. Typically, spheroids are created using various methods, such as U-bottom plates, hanging drop or semi solid media and the analysis of sphere growth has been carried out using a microscope, which is time consuming, laborious, low-throughput and lacks reproducibility. Literature has previously reported the use of an automated imager to monitor the size and growth of 3D spheroids in 96-well plates (1), where spheres were formed in 4 days from a set number of cells to return a consistent sphere size. This study highlights the use of the 384-well low attachment U-bottom plate combined with the Celigo® Imaging Cytometer to image and analyze the formation of 3D spheroids. This allows for increased throughput, number of replicates and parameters per plate as compared to 96-well plates. U87MG cells were used to create tumorspheres in 384-well plates and were subsequently analyzed by imaging. The data illustrate that reproducible 3D spheroids can be formed in 384-well plates. Fluorescent viability studies were carried out with the imager using pixel intensity analysis. Moreover, the assay was validated for drug screen using various drug compounds that have shown anti-proliferative effects. Together, these data demonstrate that the tumorsphere formation assay can be developed, validated and used for high-throughput anti-cancer compound screening in 384-well U-bottom low attachment plates using the Celigo Imaging Cytometer.

Method

U87MG cells (human glioblastoma cell line, ATCC HTB-14) were seeded at different densities per well in either in a low attachment round bottom 384-well plate (Sumitomo Bakelite Co., PrimeSurface, MS-9384U) for 3D tumorsphere formation or a TC treated, flat bottom 384-well plate (Corning 3712) for 2D adherent assay format. 2D culture media consisted of EMEM (EBSS) basal media, 10% FBS, 2mM Glutamine, 1% Non Essential Amino Acids, 1mM Sodium Pyruvate (Gibco). 3D culture media consisted of EMEM (EBSS) basal media, 2mM Glutamine, 1% Non Essential Amino Acids, 1mM Sodium Pyruvate + 1X B27, 20ng/mL EGF, 20ng/mL bFGF. Spheres were formed in 4 days from a set number of cells to return a consistent sphere size following methods reported in literature (1). The Celigo® Imaging Cytometer was used to image and analyze the formation of 3D spheroids and the 2D adherent cultures. The tumorspheres were monitored over a period of 21 days and the imager reported quantitative measurements from the brightfield images, including size and kinetic proliferation of the spheres and confluence of adherent cells. Media replenishment was performed on the same days as imaging, with a 1:1 replacement of fresh media. Diameter for 3D spheres and confluence area for 2D cultures were reported. Assay validation was performed with treating the cells with 17-AAG and imaging over time. For monitoring health, Life Technologies Calcien AM (C3100MP), Propidium Iodide (P3566), and Hoechst 33342 (H3570) were used on measurement days and imaged for intensity analysis. The assay was validated for drug screening, using 4 drug compounds in one plate, 17-AAG, Paclitaxel, Temozolomide (TMZ), and Doxorubicin that have shown anti-proliferative effects.

Results

Effect of Plating Density on Sphere Size and Confluence Measurement

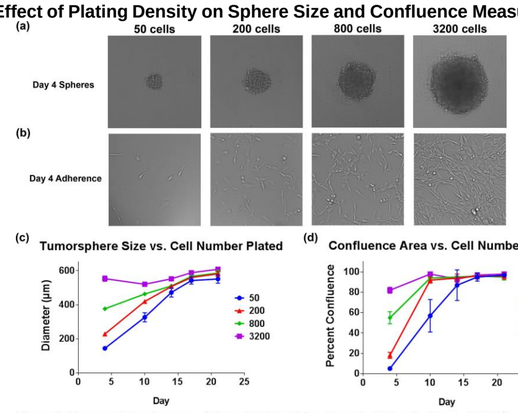

Figure 1. Representative images of 2D and 3D U87MG cultures in 384-well plates. U87MG cells were plated at densities of 50, 200, 800, and 3200 per well and imaged after 4 days on the Celigo. Cultures in U-bottom low attachment plate formed tumorspheres of various sizes (a) while cells growing TC-treated flat-bottom plates exhibited various confluence measurements (b). The Celigo was used to monitor growth and track sphere diameter in the 3D assay (c) and confluence in the 2D assay (d) over a 21 day period.

- Comparison of 2D and 3D culture growth rates demonstrated similar patterns in flat and U-bottom 384-well plates.
- Brightfield imaging on the Celigo can be successfully used for monitoring 2D and 3D proliferation assays.

3D Assay Validation in 384 well U-bottom Plate with 17-AAG

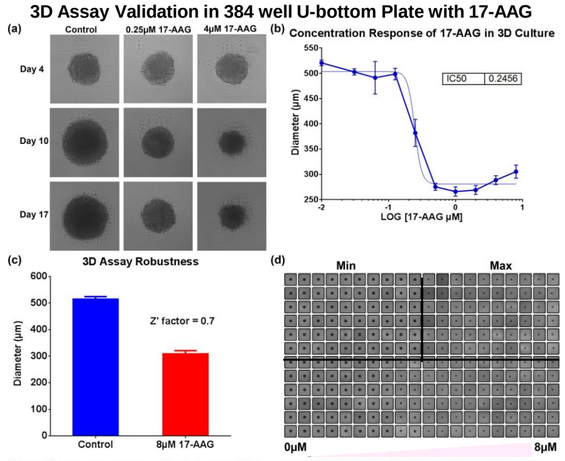

Figure 2. Anti-proliferation 3D assay validation. Tumorspheres were treated on day 4 with varying concentrations of 17-AAG in 384-well U-bottom plates. Brightfield images were taken overtime with the Celigo imager and analyzed for sphere size. Images of spheres treated with control (0.01% DMSO), 0.25µM, or 4µM 17AAG were acquired on day 4, 10, and 17 (a). A concentration response of 17-AAG on sphere size measured an IC_{50} value of 0.25µM on day 10 (n=12) (b). Z' factor of 0.7 was calculated using control and 8µM 17-AAG treatment on spheres (n= 60) (c). Plate map view of the software visualizes control quadrant (upper left), 8µM treated quadrant (upper left) and 17-AAG concentration response curve (lower half) (d).

- A 3D culture proliferation assay for drug screening was validated with a 384-well low attachment U-bottom plate.
- Visual observation of sphere diameter indicated that tumorsphere growth was inhibited by 17-AAG at 0.25µM and sphere size was reduced at 4µM.
- The use of 384-well plates allowed for an increased number of wells (as compared to a 96-well plate) and provided strong assay statistics with a Z' factor of 0.7 and an IC_{50} of 0.25µM.

2D Assay Validation in 384-well TC-treated Plate with 17-AAG

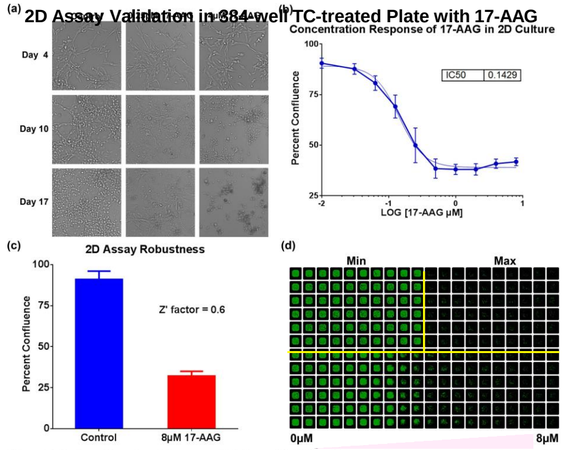

Figure 3. Anti-proliferation 2D assay validation. U87MG adherent cells were treated on day 4 with varying concentrations of 17-AAG in 384 well TC-treated flat-bottom plate. Brightfield images were taken overtime with the Celigo and analyzed for confluence. Images of control, 0.25µM, 4µM 17-AAG treated cells on day 4, 10, and 17 (a). A concentration response of 17-AAG measuring confluence on day 10 gave an IC_{50} value of 0.14µM (b). Z' factor of 0.6 was calculated using control, and 8µM 17-AAG treatment on plate (n=60) (c) The plate map view of Celigo software using a "green fill area" that signifies well confluence (d).

- A 2D culture proliferation assay was validated for drug screen in a 384-well TC-treated flat bottom plate.
- Visual observation of well confluence indicated that U87MG cell growth can be inhibited by 17-AAG at 0.25µM. Images also show that at higher concentrations, 17-AAG induced cell aggregation.
- The use of 384-well plates allowed for an increased number of wells (as compared to a 96-well plate) and provided strong assay statistics with a Z' factor of 0.6 and an IC_{50} of 0.15µM.
- Comparison of 2D and 3D cultures demonstrated that while having similar effect, a higher concentration of 17-AAG was required in 3D cultures.

Viability of 17-AAG on 3D and 2D Cultures

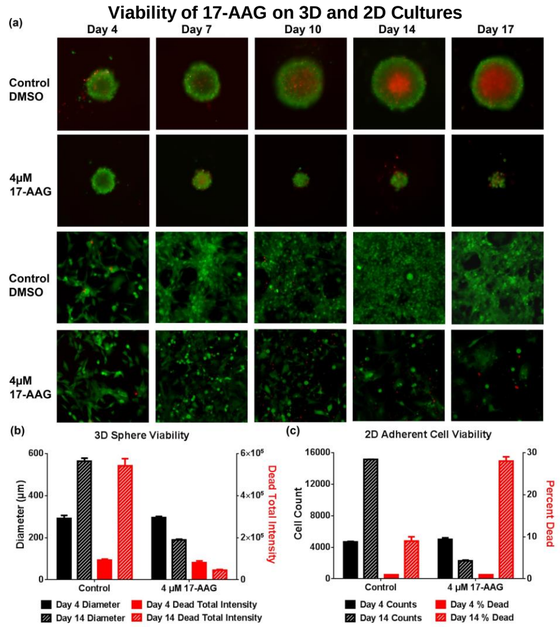

Figure 4. Viability measurements of 2D and 3D cultures. Cells were stained with Calcein AM (green), Propidium Iodide (red) and Hoechst (blue) to measure for live and dead cells on days 4, 7, 10, 14, and 17 and imaged on the Celigo. Spheres and cell nuclei were identified and fluorescent intensity measurements were taken with the identified area. 2D and 3D representative images of cell and sphere viability (a). Celigo measurements of diameter and dead total intensity for 3D assay (b). Cell counts and percentage of dead cells for 2D assay (c).

- While tumorsphere size increased overtime, a higher number of dead cells were observed in the tumorsphere core.
- Spheres treated with 17-AAG decreased in size but maintained viable cells with no significant increase of dead cells.
- 17-AAG decreased cell counts in 2D culture and induced a significant amount of cell death.

Drug Screening in 2D and 3D Assay Format

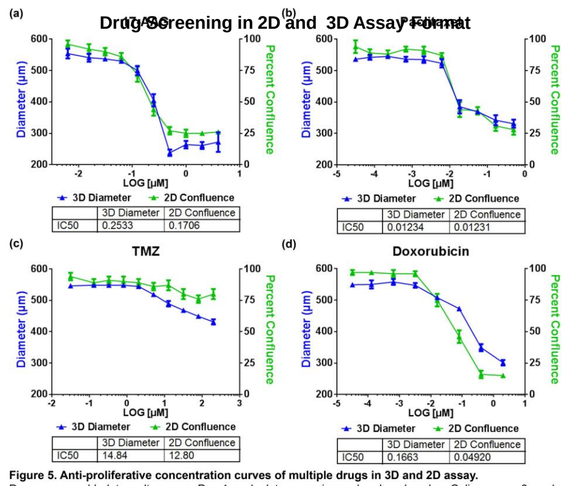

Figure 5. Anti-proliferative concentration curves of multiple drugs in 3D and 2D assay. Drugs were added to cultures on Day 4 and plates were imaged and analyzed on Celigo over a 3 week period. Graphs of day 14 for 17-AAG (a), Paclitaxel (b), Temozolomide (TMZ) (c), Doxorubicin (d).

- Concentration response curves with four drugs were successfully achieved for both 2D and 3D assays. Use of 384-well plate increased screening numbers and replicates per plate.
- As previously stated, 2D and 3D assays exhibited similar patterns in tumorsphere size and confluence. Higher concentration of drugs in 3D assays were required to produce the same effect as in 2D.

Effect of Drugs on 2D and 3D Culture Viability

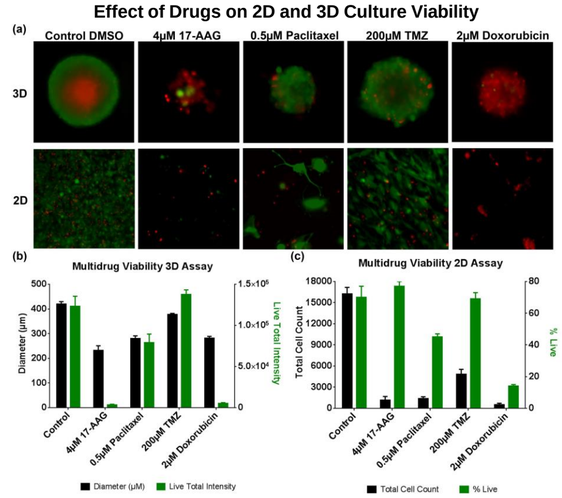

Figure 6. Drug treatment outcome on 2D and 3D culture aspects and viability. 2D and 3D cultures were treated with multiple drugs (17-AAG, Paclitaxel, TMZ or Doxorubicin) and stained for viability after 21 days (a). 3D sphere diameter and total live fluorescent intensity (b). 2D cell count and percent live cell fluorescence (c).

- The various drugs used to inhibit 2D and 3D growth created different patterns of live and dead cells within these cultures.
- Although 17-AAG decreased sphere size and total cell counts, living cells were detected in both 2D and 3D assays.
- Paclitaxel decreased sphere size and cell counts. Adherent cell morphology analysis showed an increase in cell size (2).
- Temozolomide decreased adherent cell growth but no significant decrease in sphere size or cell death was observed (3).
- Doxorubicin decreased sphere size and total cell counts. While few living cells were detected in spheres and in 2D culture, significant cell death was observed.

Conclusion

Many traditional 2D *in vitro* anti-cancer compound studies have a low success rate when transitioning to clinical trials. 3D culture assays are showing strong evidence of how their microenvironment is more indicative of drug treatment on tumors *in vivo*. Questions still remain as to how and why 2D and 3D cultures behave differently in treatment with anti-proliferative compounds. Previously published data have reported the utility of automated imaging and analysis of tumorspheres with a 96-well U-bottom low attachment plate (1). This study demonstrates that the cell culture assay for tumorsphere size was successfully adapted to 384-well plate for a 3D spheroid assay using U-bottom low attachment 384-well and the Celigo Automated Imager for image acquisition and analysis. The use of 384-well allowed for more replicates as compared to 96-well plate format which rendered the assay suitable for high-throughput drug screening. The transition from 2D to 3D assays highlighted some differences between the effects of various common anti-cancer drugs. Specifically, higher concentrations of drugs were required to produce similar effects in 3D as compared to 2D. Moreover, the utilization of an imager provided insight on sphere and cell fate after long term drug treatment. This study demonstrates how a 3D spheroid culture assay for studying tumorsphere size was successfully adapted to 384-well U-bottom low attachment plate using the Celigo for image acquisition and analysis. Together, these data demonstrate that 3D anti-proliferation assays can be validated and used for high-throughput screening of anti-cancer compounds.

References:

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