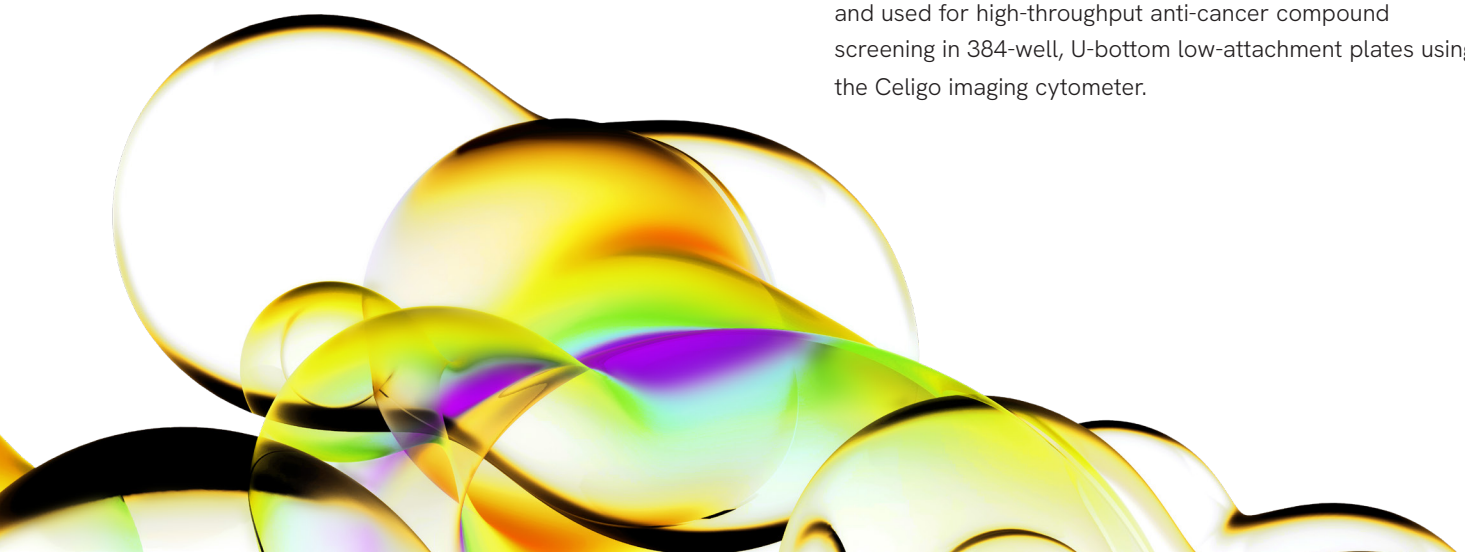
3D Tumor spheroid analysis method for HTS drug discovery using Celigo imaging cytometer.

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 lacking in 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 that 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.



Materials and methods

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 for 3D tumorsphere formation or a TC treated, flat bottom 384-well plate 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 the 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 bright field images, including size and kinetic proliferation of the spheres and confluence of adherent cells.

Media replenishment was performed on the same days as imaging. 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, Propidium Iodide, and Hoechst 33342 were used on measurement days and imaged for intensity. The assay was validated for drug screening, using four anti-proliferative drug compounds in one plate: 17-AAG, Paclitaxel, Temozolomide (TMZ), and Doxorubicin.

Results

Effects of plating density of spheroid size and confluence measurement

- 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.

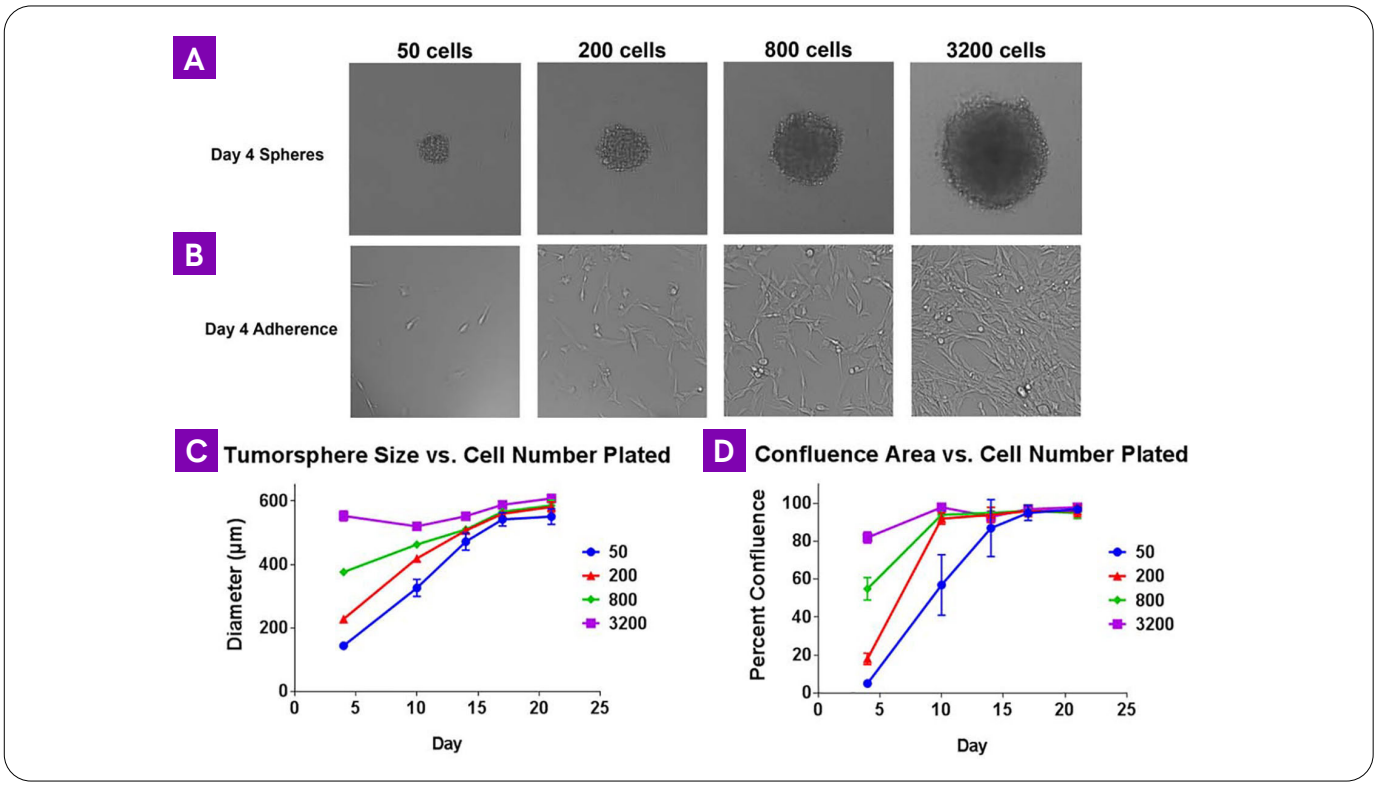


Figure 1. Representative images of 2D and 3D U87MG cultures in 384-well plates.

U87MG cells were plated at densities of 50, 200, 800, and 3,200 cells per well and imaged after four days on the Celigo. (A) Cultures in U-bottom, low-attachment plate formed tumorspheres of various sizes, (B) while cells growing in TC-treated, flatbottom plates exhibited various confluence measurements. The Celigo was used to monitor growth and track sphere diameter in the (C) 3D assay and confluence in the (D) 2D assay over a 21-day period.

3D assay validation in 384-well, U-bottom plate with 17-AAG

- 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 as 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.

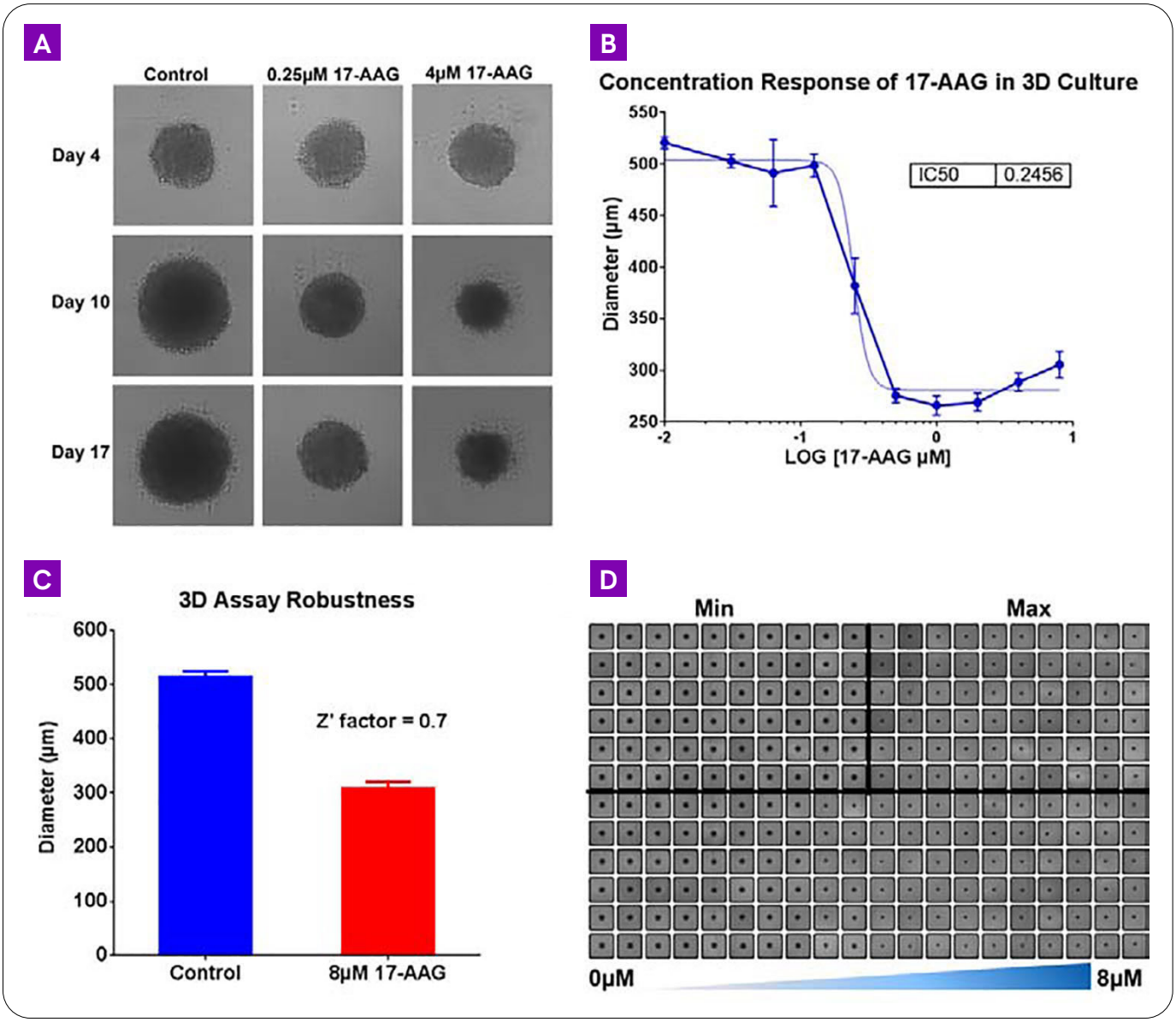


Figure 2. Anti-proliferation 3D assay validation.

Tumorspheres were treated on day 4 with varying concentration of 17-AAG in 384-well, U-bottom plates. Brightfield images were taken over time with the Celigo imager and analyzed for sphere size. (A) Images of spheres treated with control (0.01% DMSO), 0.25 µM, or 4 µM 17-AAG were acquired on days 4, 10, and 17. (B) A dose response of 17-AAG on sphere size measured an IC_{50} value of 0.25 µM on day 10 (n=12). (C) Z′ factor of 0.7 was calculated using control and 8 µM 17-AAG treatment on spheres (n=60). (D) Plate map view of the software visualizes control quadrant (upper left), 8 µM treated quadrant (upper right), and 17-AAG dose response curve (lower half).

2D assay validation in 384-well TC-treated plate with 17-AAG

- 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.

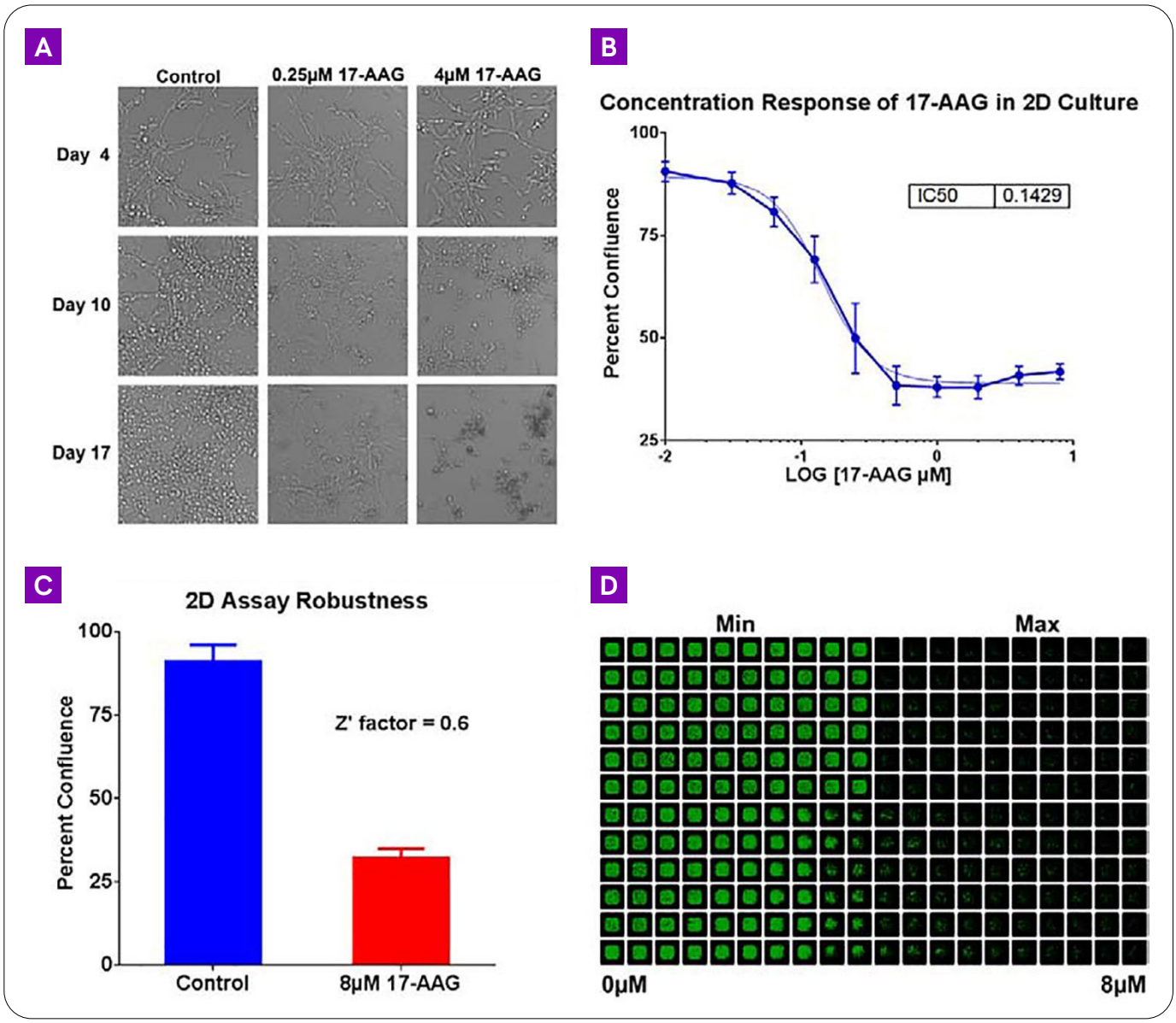


Figure 3. Anti-proliferation 2D assay validation.

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 over time with the Celigo and analyzed for confluence. (A) Images of control, 0.25 µM, 4 µM 17-AAG treated cells on days 4, 10, and 17. (B) A concentration response of 17-AAG measuring confluence on day 10 gave an IC_{50} value of 0.14 µM. (C) Z′ factor of 0.6 was calculated using control and 8 µM 17-AAG treatment on plate (n=60). (D) The plate map view of Celigo software using a "green fill area" that signifies well confluence.

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

- While tumorsphere size increased over time, 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 in dead cells.
- 17-AAG decreased cell counts in 2D culture and induced a significant amount of cell death.

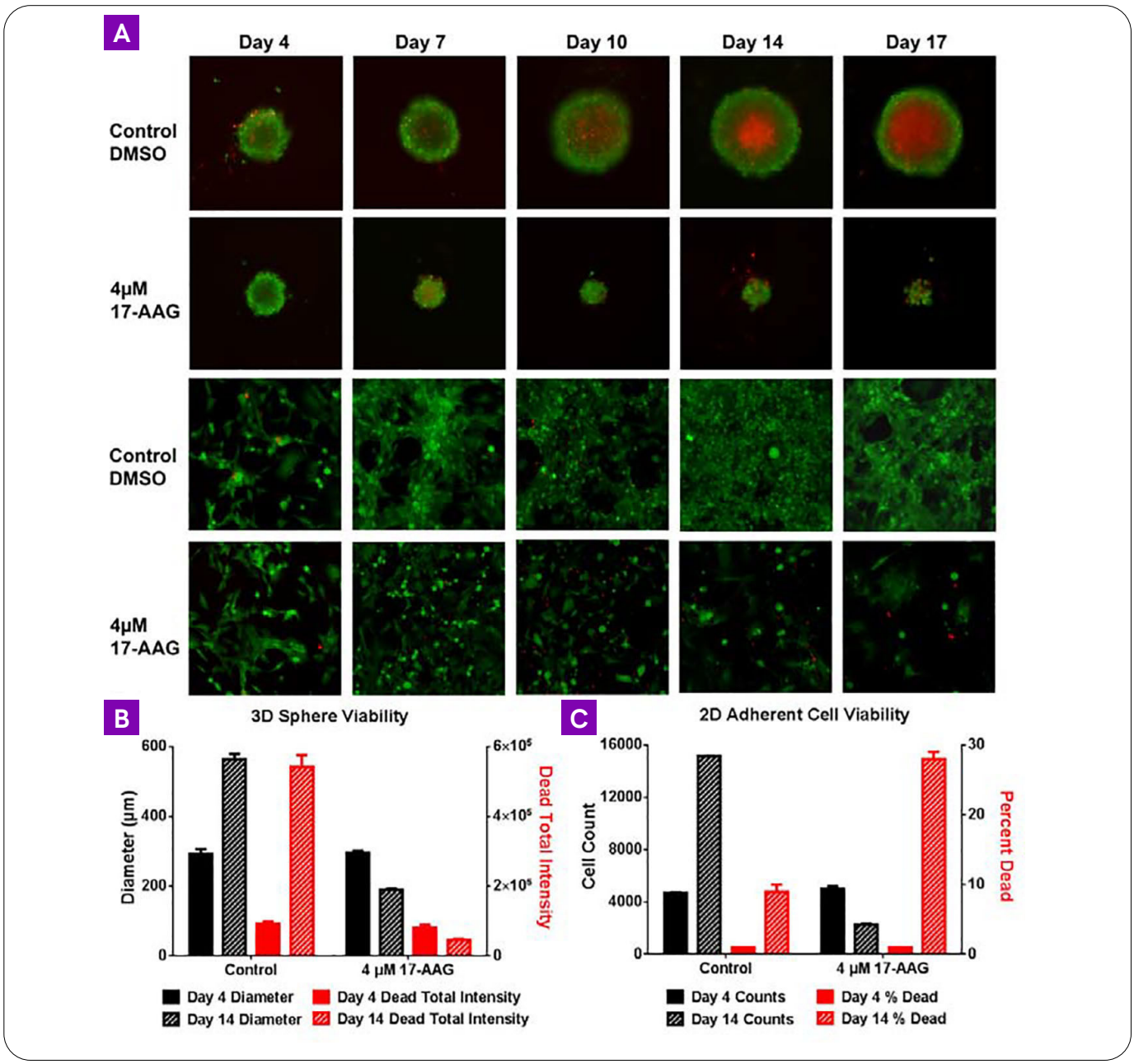
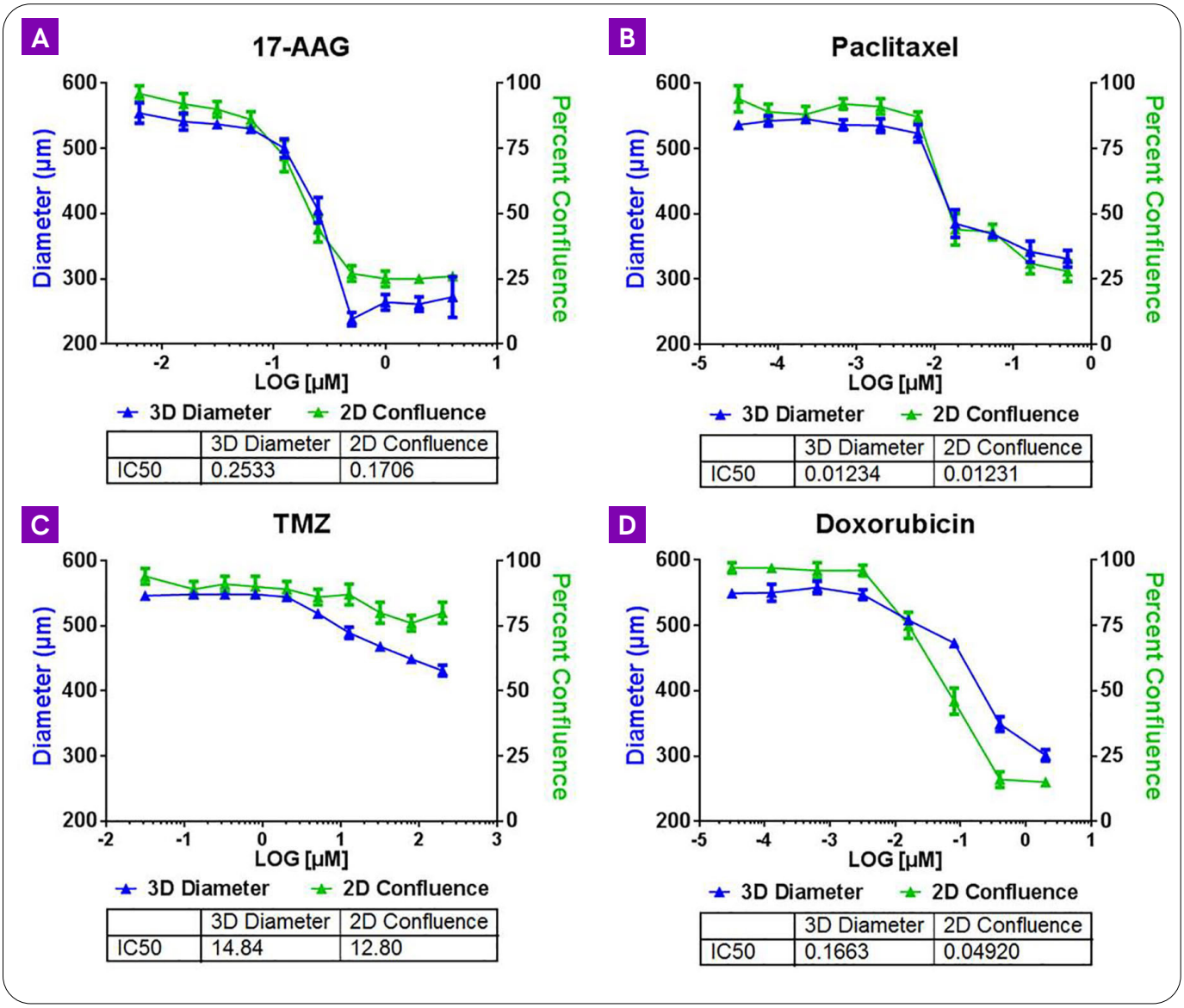


Figure 4. Viability measurements of 2D and 3D cultures.

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 with Celigo. (A) 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. (B) Celigo measurements of diameter and dead total intensity for 3D assay. (c) Cell counts and percentage of dead cells for 2D assay.

Drug screening in 2D and 3D assay format

- 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 concentrations of drugs in 3D assays were required to produce the same effect as in the 2D samples.



	3D Diameter	2D Confluence
IC50	0.2533	0.1706

	3D Diameter	2D Confluence
IC50	0.01234	0.01231

	3D Diameter	2D Confluence
IC50	14.84	12.80

	3D Diameter	2D Confluence
IC50	0.1663	0.04920

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 (A) 17-AAG, (B) Paclitaxel, (C) TMA, and (D) Doxorubicin.

Effect of drug on 2D and 3D sample viability

- 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.

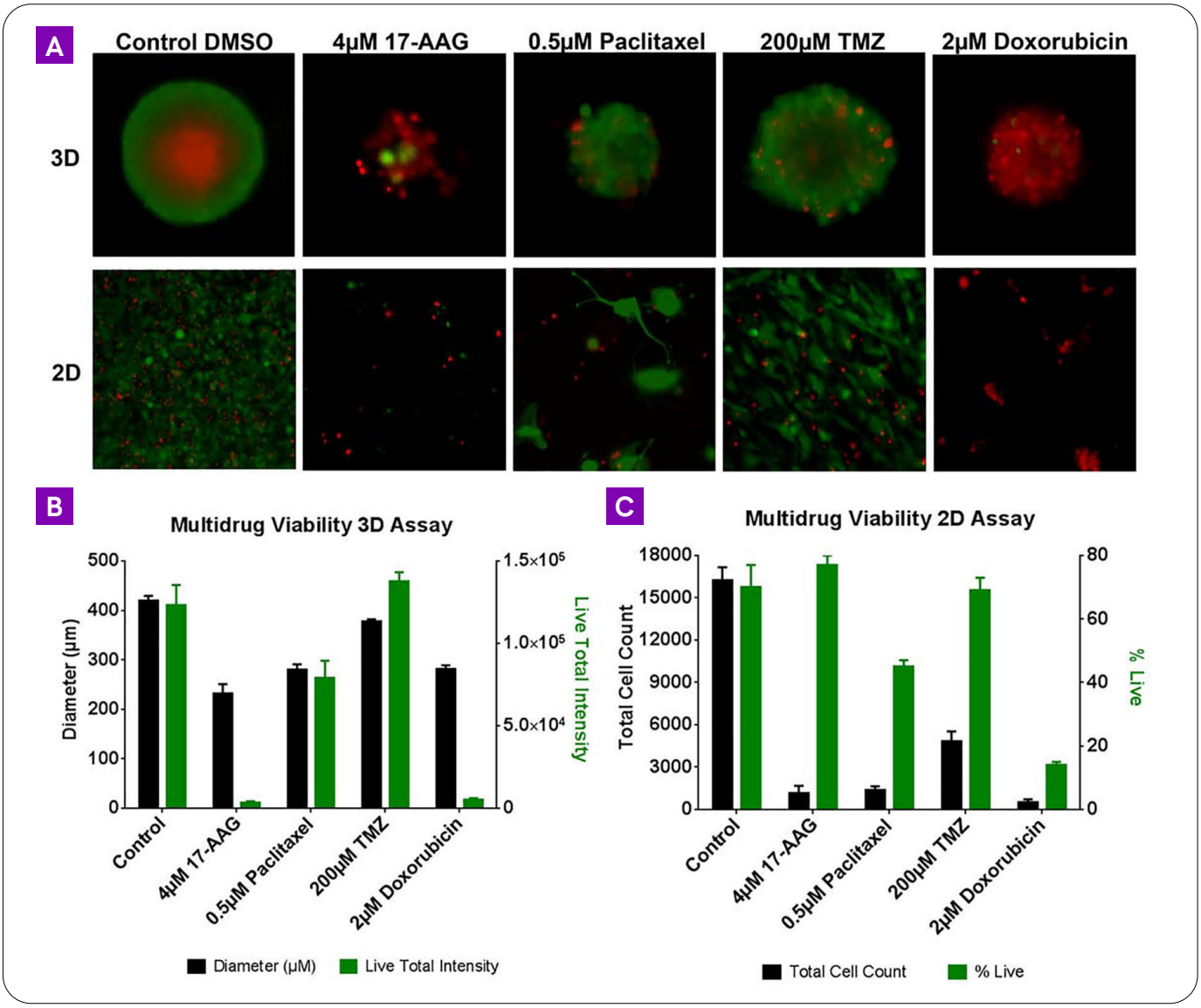


Figure 6. Drug treatment outcome on 2D and 3D culture aspects and viability.

(A) 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. (C) 2D cell count and percent live cell fluorescence.

Conclusions

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 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 antiproliferative 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].

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 samples as compared to 2D samples. 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 antiproliferation assays can be validated and used for high-throughput screening of anti-cancer compounds.

References

1. Vinci, M., et al., Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biol,* 2012. 10: p. 29.

2. Mikhail, A.S., S. Eetezadi, and C. Allen, Multicellular tumor spheroids for evaluation of cytotoxicity and tumor growth inhibitory effects of nanomedicines *in vitro*: a comparison of docetaxel-loaded block copolymer micelles and Taxotere(R). *PLoS One,* 2013. 8(4): p. e62630.

3. Gunther, W., et al., Temozolomide induces apoptosis and senescence in glioma cells cultured as multicellular spheroids. *Br J Cancer,* 2003. 88(3): p. 463-9.