APPLICATION NOTE

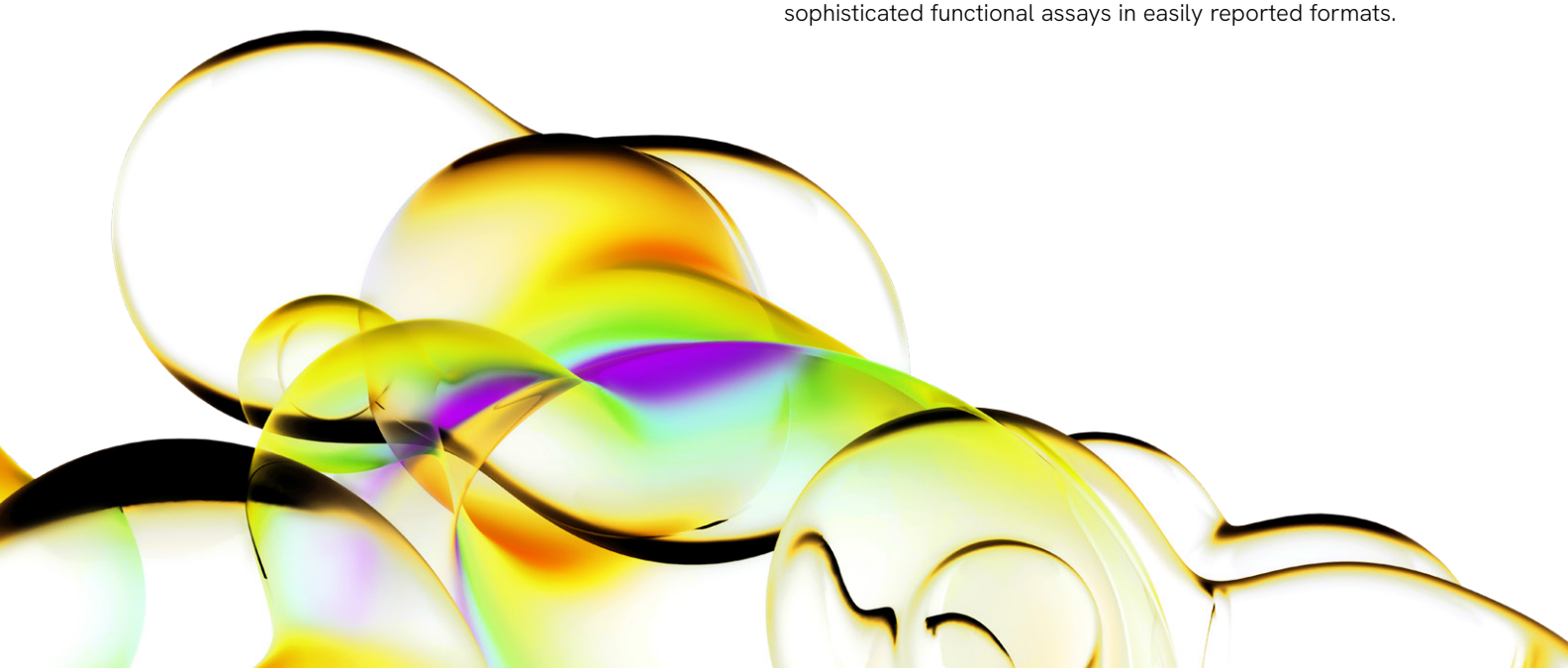
Non invasive spheroid analysis and characterization.

Introduction

The current 2D methods for cancer drug discovery have not been as successful at identifying drug candidates as expected. In an attempt to overcome this challenge there has been an explosion of research in 3D tissue culture that facilitated the development of new *in-vitro* tumor model assays. Historically, the hanging drop technique[1] was used to create embryoid bodies and more recently cancer spheroids. Today, multiple techniques such as agarose overlay assays[2], spinner flasks[3], low binding plates[4], polyHema coated plates[5], round bottom ultra low attachment plates[6] and micro-patterned plates[7] are used to generate spheroids.

The above techniques have both advantages and disadvantages. Numerous questions are still to be answered and the scientific community has yet to settle on a single method for routine research or drug evaluation. Should we be creating clonal spheroids or spheroids from different cell types? Should we be screening using single uniform spheroids or large numbers of spheroids of various sizes? Furthermore, the ability to detect, analyze and characterize these spheroids in a simple and efficient manner is also needed to bring these manual, user dependent assays to a level that can be used for high throughput assays.

The Celigo® imaging cytometer is the ideal solution for 3D spheroid measurements, both quantitative and qualitative. Its unique brightfield capabilities allow the user to quickly and easily identify every spheroid in every well automatically. Revvity's state of the art software reports on many parameters allowing spheroid characterization, and the development of more sophisticated functional assays in easily reported formats.



Approach and results

MDA-MB-231 and MCF-7 cells were grown on fl at, low attachment 96 well plates for 16 days. The various sized spheroids produced from these cultures were analyzed on days 2, 5, 9 and 16 using the Celigo's colony counting application: Tumorsphere (TS) application (Fig 1A-C). Brightfield imaging and segmentation was performed to detect each spheroid in one Field of View (FOV) or as a whole well image (1B). The TS application automatically reports the number, size and shape of the spheroids and the analysis software can easily separate small pieces of debris from spheroids. Not only can the Celigo detect the various sizes of the TS's, it can also easily track the growth of cultures over several days, as depicted in the graph in Fig 1C.

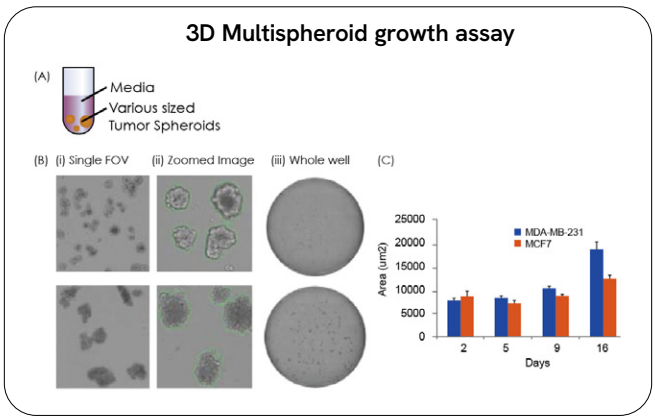


Figure 1: (A) Schematic of the plate set up for producing multiple tumor spheres in low attachment plate (B) Brightfield images of MCF-7 (top) and MDA-MB-231 (bottom). (i)Tumorsphere cultured on low attachment culture plates (left images). (ii) Zoomed in image of tumorsphere segmentation allowing analysis and characterization (middle images). (iii) Whole well images from a 6 well plate (right image). (C) Graphical analysis of MCF-7 and MDA-MB-231 tumorsphere growth tracking experiment using area over 16 days.

More recently, Vinci et al developed a micro plate method for generating reproducibly sized single spheroids in each well of a 96 well round bottom plate. Using this method, spheroid development from U-87 MG, a human adult glioma cell line, was analyzed over 14 days (Fig 2A). At day 4 post initiation, TS were treated with 17-AAG (17-(allylamino)-17¬demethoxygeldanamycin), an HSP90 chaperone inhibitor, over a range of concentrations. The plates were imaged using brightfield illumination and analyzed on the Celigo image cytometer over the time course of 14 days. The graphical data (Fig 2C) demonstrates a clear concentration-dependent inhibition and this is confirmed using the whole plate image view (Fig 2B). The excellent reproducibility that can be observed across the 6 replicates has an intra-plate CV of between 5.53 and 7.28%.

The combination of the micro plate based assay and the ease of use of the Celigo imaging cytometer led Vinci et al to develop and validate further spheroid based functional assays. Using one of the many other Celigo applications, confluence detection, Vinci et al were able to use the Celigo imaging cytometer for the first time to perform functional readouts from these spheroids in a non-invasive manner.

This first such assay is a readout of cellular invasion of cells from the spheroids into a matrix e.g. matrigel. U-87 MG human adult glioma cells were seeded into round bottom low attachment 96 well plates. At day 4 post initiation, the spheroids were embedded in matrigel and treated with a range of concentrations of 17- AAG. Using the confluence application, brightfield imaging and segmentation were performed to detect the change in area of invasion over 3 days in the presence or absence of drug. The graphs and images demonstrate the inhibition of invasion of the U-87 MG cells in the matrigel by 17-AAG (Fig 3B and C).

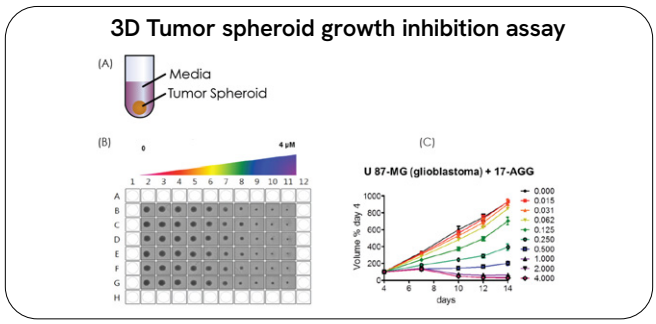


Figure 2: (A) Schematic of the plate set up for producing individual tumor spheroids in a round bottom ultra low attachment plate. (B) Brightfield whole plate image from Day 14 of the spheroid growth tracking experiment demonstrating concentration-dependent inhibition of spheroid growth using 17-AAG, an HSP90 chaperone inhibitor. (C) Quantitative analysis of U-87 MG spheroid growth inhibition by 17- AGG over 14 days.

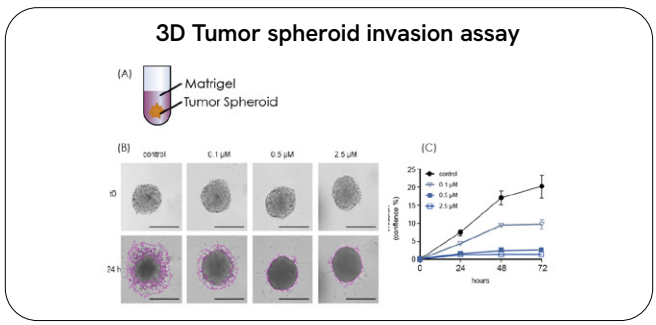


Figure 3: (A) Schematic of the plate set up for producing U-87 MG tumor spheroid invasion assay in round bottom ultra low attachment plates (B) Brightfield whole well images of the 0 and 24 hr time points of the invasion of U-87-MG cells with and without invasion segmentation. It is clear that there is a concentration-dependent inhibition of invasion, scale bar is 500um. (C) Graphical analysis of U-87 MG spheroid invasion assay over 72-hours using 17-AAG, an HSP90 chaperone inhibitor over 72 hours.

The second functional assay examines migration/haptotaxis of cells from the spheroid and again uses the confluence application. U-87 MG spheroids were generated in round bottom low attachment 96-well plates as before. At day 4 post initiation, the spheroids were transferred to a flat bottom 96-well plate previously coated with gelatin. Migration /haptotaxis of U-87 MG tumor cells from the spheroid across the gelatin substrate was analyzed using the confluence application on the Celigo imaging cytometer. Image analysis and segmentation of the spheroid cells and the increase or decrease in confluence area over time with and without 17- AAG treatment is shown in Fig 4B and C. The graphical data and the representative images obtained on the Celigo at 24 hours both demonstrate that the Celigo can detect the reduction in migration/ haptotaxis induced by drug 17-AAG. It is of note all of the data presented here is generated without the addition of any dyes or probes and thus these assays are truly dynamic, non-invasive and can be repeated numerous times without harming the cells.

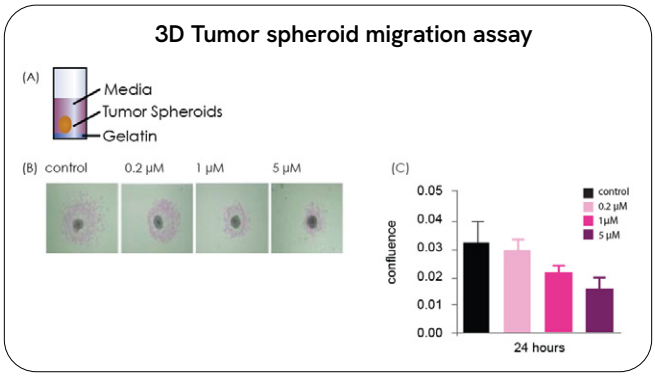


Figure 4: (A) Schematic of the set up for producing a U-87 MG tumor spheroid migration assay using gelatin coated tissue culture plates. (B) Segmented brightfield well images of the migration of U-87 MG at the 24 hr time point .17-AAG inhibits the migration of U-87 MG cells on gelatin coated plates in a concentration-dependent manner. (C) Quantitative analysis of U-87 MG spheroid migration over 24-hours using 17-AAG, an HSP90 chaperone inhibitor.

Conclusions

The Colony Counting: Tumorsphere application on the Celigo image cytometer provides an efficient, reproducible automated method for assessing the number, size and shape of spheroids within both flat and round bottom multiwell plates. The Tumorsphere application has also been used for analyzing neurosphere and mammosphere cultures as well as spheroids in agarose overlay and hanging drop technologies. The functional analysis of spheroids using the confluence application within the Celigo brings an important added dimension, allowing researchers to monitor not only spheroid initiation but also to obtain dynamic, non invasive measurements of growth, cell migration, cell invasion and assess the response of these key hallmarks of cancer to drug treatment.

References

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