# Combination of Ultra-Low Attachment Spheroid Plates with a Tissue Clearing Technique for Easy-to-Use 3D Cell Culturing and True 3D Characterization.

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## Abstract

In the last few years there has been a substantial increase in the use of three-dimensional (3D) cell culture models in the drug discovery process since these models more accurately mimic the *in vivo* environment compared to traditional monolayer cultures. However, the rapid adoption of 3D cell culture models for screening purposes has been slowed by the lack of robust and easy-to-use culturing platforms and the difficulties associated with imaging and characterization of 3D cell culture models (e.g. spheroids, microtissues, organoids). Through this work, we evaluated the use of Corning® Ultra Low Attachment Spheroid Microplates for *in situ* formation of spheroids. HepG2 hepatocyte spheroids of 300 µm in diameter were rapidly and reproducibly generated; cell viability was determined by employing live/dead molecular probes to construct dose-response curves of antiproliferative compound paclitaxel. Furthermore, we evaluated the use of the tissue clearing technique Visikol® HISTO-M™ coupled with high content confocal imaging. It was shown that the combination of these technologies allowed for the rapid and automated 3D analysis of spheroids in a whole-mount well-plate format. It was shown that the application of a tissue clearing technique to 3D cell culture characterization resulted in a 3-fold increase in cells detected, and further allowed for the analysis of dose response as a function of location within the spheroid to detect differentiated effects in the inner and outermost cells.

## Materials and Methods

HepG2 cells were cultured according to published techniques. Wells of Corning ULA U-bottom plates (Corning Cat. No. 4515) were seeded with 1,000 cells, and incubated with 5% $CO_2$ at 37°C for 2 days to form spheroids.

**Treatment of spheroids with antiproliferative compound**

Spheroids were treated with antiproliferative compound (paclitaxel) on day 0, and again on day 3. Paclitaxel was dissolved in DMSO, and from this stock solution, 10-fold serial dilutions were prepared to make 100x working dilutions. Compound was diluted to final assay concentration in growth media. The assayed concentrations were 1 µM, 500 nM, 100 nM, 10 nM, 1 nM, and vehicle control.

**Fixation and immunolabeling**

On day 5, spheroids were fixed using 10% NBF, followed by washing in PBS to remove fixative. Spheroids were treated with methanol, followed by 20% DMSO/methanol to improve penetration of antibodies and stains. Spheroids were blocked with 10% donkey serum. Spheroids were incubated with rabbit anti-Ki67 antibody (1:150 dilution) to label proliferating cells. Nuclei stained with DAPI (ThermoFisher).

**Clearing and high throughput imaging of spheroids**

Spheroids were cleared with Visikol HISTO-M. Imaging of spheroid plate was accomplished using a GE InCell 6000 High Content Confocal Imager. Z-stacks were collected for each tissue, using 5 µm steps. Images were processed using ImageJ, and DAPI+ and Ki67+ cells were counted using CellProfiler.

## Results and Discussion

**Visikol HISTO-M enables visualization of spheroid interior**

As seen in Figure 2A, when imaging 3D tissue cultures, the interior of the spheroid appears dark, as light scattering drastically reduces signal due to the opacity of the spheroids. Using Visikol HISTO-M, light scattering was greatly reduced, allowing for comprehensive profiling of the interior of the spheroids, shown in Figure 2B.

**Visikol HISTO-M increases detectable cells in interior**

CellProfiler was used for automated cell-counting of the confocal image stacks. The data shown in Figure 3 illustrate the inherent problems with imaging non-cleared spheroids. The image stack progresses into the non-cleared spheroid, fewer and fewer cells are detected in each plane, until only the periphery is detectable. This is due to light scattering caused by the opacity of non-cleared spheroids, which limits imaging to approximately 20-50 µm, even with confocal microscopy. With cleared spheroids (Figure 3) cells are detectable across the entire image plane, deep into the spheroid. As can be seen in the graph, on average, 3-fold more cells were detected on each plane of the cleared spheroid than the non-cleared spheroid. The effect is even more dramatic deeper in the spheroid; at 120 µm 7-fold more cells were detectable.

When conducting confocal imaging of spheroids without clearing, only the outermost cells are detected due to light scattering reducing signal. Use of a tissue clearing agent greatly increases the number of cells detectable by high content imaging. Corning ultra-low attachment plates are chemically compatible with Visikol HISTO-M clearing, so generation of spheroid, labeling, and imaging can be conducted without transfer of spheroids. The clearing process takes only minutes, and is done within the wells.

**Case study: Antiproliferative assay on HepG2 spheroids**

Paclitaxel treated and control spheroids were imaged using a GE InCell 6000 High Content Imager to obtain multicolor image stacks with a 5 µm z-step size. Ki67 was used as a marker for proliferation. Cells were counted automatically with CellProfiler, giving total cell counts (DAPI+) and Ki67+ cell counts. Ki67+ cells were counted only if they colocalized with DAPI+ staining. Ki67%, the ratio of Ki67+ to total cells, was used as the measurement of proliferation. Dose response curves were constructed for cleared and non-cleared spheroids, shown in Figure 4. As can be easily discerned from Figure 4A, there was a significant measured difference between dose response curves constructed from cleared and non-cleared spheroids. The non-cleared spheroids demonstrated significantly higher apparent Ki67%--the outer layers of cells were the only detectable cells in non-cleared spheroids, and this population of cells exhibits a higher relative level of proliferation compared to the inner population of cells, as is reflected in the dose response curves for cleared spheroids. Since the entire cell population is measured in cleared-spheroids, the Ki67% measured is more representative of the spheroid population, since the inner, less-proliferative cells are included in measurements.

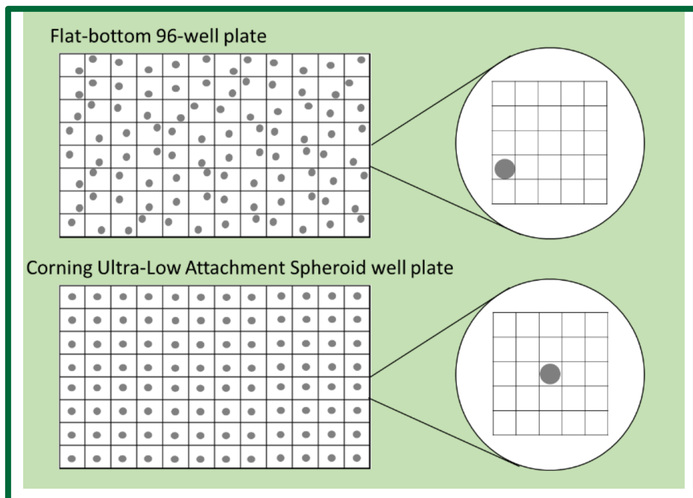


**Figure 1.** Conventional flat-bottom 96-well plates result in randomly placed spheroids, thus a much larger area must be scanned by the high content imager to find the spheroid to image, reducing throughput. Corning ULA U-bottom 96-well spheroid plates center the spheroids permitting accelerated image acquisition.

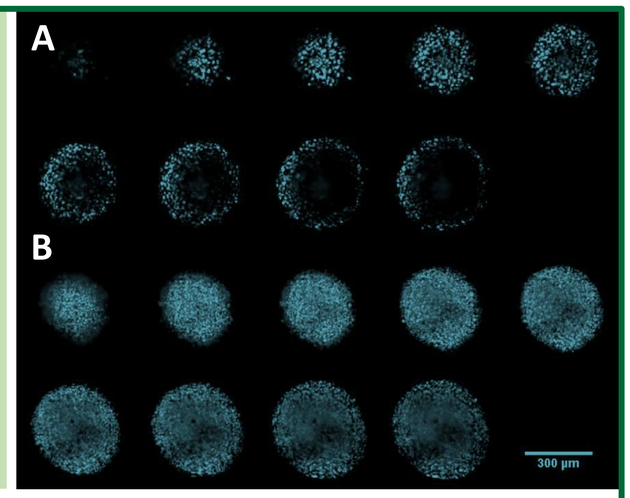


**Figure 2.** Montage of 20 µm slices from confocal image stack of nuclear-stained spheroid; A) Non-cleared spheroid; B) Cleared spheroid


**Figure 3.** Cells detected at various z-depth through cleared and non-cleared HepG2 spheroids.

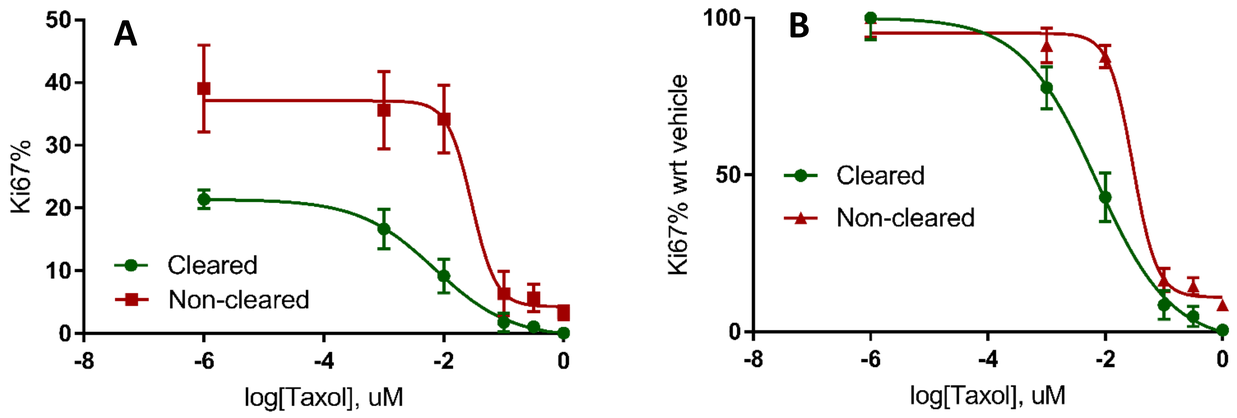


**Figure 4.** Ki67% dose response curves for cleared and non-cleared HepG2 spheroids. A) Absolute Ki67% dose response curve for paclitaxel-treated HepG2 spheroids; B) Dose response curves showing Ki67% with respect to vehicle Ki67% for paclitaxel-treated HepG2 spheroids;

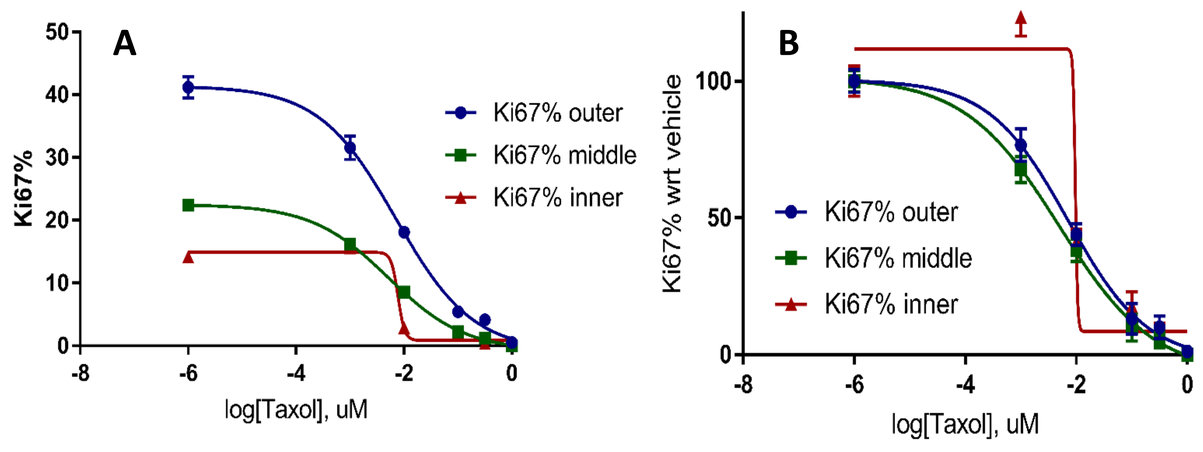


**Figure 5.** Spatial dose response curves for Ki67% measured in HepG2 spheroids. A) Absolute Ki67% dose response curves for three populations of cells in paclitaxel-treated HepG2 spheroids; B) Dose response curves showing Ki67% with respect to vehicle Ki67% for three populations of cells in paclitaxel-treated HepG2 spheroids;

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