Clearing with Visikol® HISTO-M™ enables more accurate quantitation of viability for distinguishing a necrotic core from treatment-induced cell death

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Introduction

Recent advances in tissue culture technology have made the adoption of 3D cell culture models to study a number of different disease states and associated therapeutic approaches in a high-throughput format more widely accessible. For example, multicellular spheroids have been generated from cancerous cells to screen potential chemotherapeutic agents[1], hepatocytes to screen for drug-related liver toxicity[2], and even isolated primary cells from the human cortex to study drug penetration across the blood brain barrier[3]. Given the crucial nature of cell viability in assaying the cytotoxic effects of therapeutic candidates in these various contexts, a reliable determination of the frequency and distribution of non-viable cells is necessary for the establishment of a baseline in newly developed models and for determining the localization of cytotoxic effects in treated models[1].

In this work, we employed the commonly used hepatocellular carcinoma line, HepG2 to generate multi-cellular spheroids of varying sizes using Corning® Ultra-low Attachment Spheroid Micro-plates. Combined with ThermoFisher's LIVE/DEAD™ Fixable Green Dead Cell Stain and spheroid clearing with Visikol HISTO-M we were able to determine the frequency and localization of non-viable cells. Additionally, we demonstrated that treatment with a cytotoxic compound, taxol, induces a spatial distribution of cell death that is distinct from that of a necrotic core. The ability to distinguish between these modes of cell death may prove crucial in high-throughput drug screening assays, particularly those involving larger-scale 3D culture models.

Methods

HepG2 cell culture and treatment

HepG2 cells were maintained in Advanced DMEM, supplemented with 5% fetal bovine serum, 1x GlutaMAX and 1x antibiotic-antimitotic in a humidified, 37°C, 5% CO_2 incubator and passaged via light trypsinization with 0.05% trypLE and 1 mM EDTA upon reaching 80% confluence. Trypsinized cells were resuspended in complete medium and 5 x 10^2 - 16 x 10^3 cells were plated in each of 96 wells of a Corning Round Bottom Ultra Low Attachment Spheroid Microplate. For taxol-treated plates, 1,000 cells were seeded per well. All spheroids were maintained under standard culture conditions for 4 days, exchanging half of the media volume with fresh complete media after 2 days. For taxol-treated plates, half of the media volume was replaced with paclitaxel diluted in complete culture medium on day two, and treatment proceeded for an additional 48 h.

Staining and fixing HepG2 spheroids

Spheroids were washed twice with 1X D-PBS, and a 1:1000 dilution of Thermo LIVE/DEAD Fixable Green Dead Cell Stain (reconstituted according to manufacturer's instructions and diluted in 1X D-PBS) was added to each well. Following 45 minutes of room temperature incubation with the LIVE/DEAD staining solution, spheroids were washed twice with 1X D-PBS, fixed with 10% neutral buffered formalin and permeabilized with 0.2% Triton, each for 30 min at room temperature. Fixed spheroids were then stained with a 1:5000 dilution of DAPI in Visikol Antibody Buffer for 1 h at room temperature.

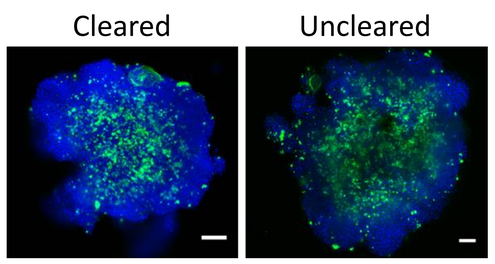
Clearing and high throughput imaging of spheroids

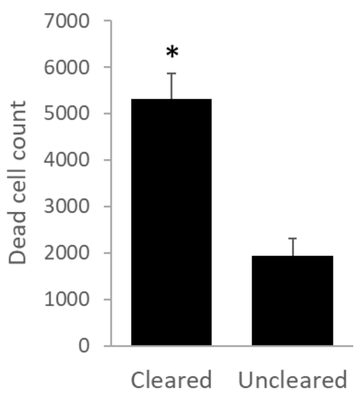
Stained spheroids were washed twice with Visikol washing buffer, once with deionized water, once with 50% methanol in deionized water, and once with 100% methanol. For clearing, as much methanol as possible was removed from the well, and Visikol HISTO-M was added to each well for subsequent imaging on a CX7 LZR High Content Confocal Imager. Z-stacks were collected for each spheroid, using 10 µm steps. Image analysis was performed using both custom ImageJ macros and CellProfiler.

Results

Optical clearing with Visikol HISTO-M facilitates higher cell frequency detection and reveals a necrotic core

When high frequencies (16 x 10^3) of HepG2 cells were seeded in ultra-low attachment spheroid forming plates, cells at the core of the spheroid become difficult to distinguish and thus quantify. However, clearing enables acquisition of a clearer image of the spheroid interior (left vs right, above; scale bar, 100 µm). As a result, more non-viable cells are detectible and quantifiable via automated image processing algorithms in cleared versus uncleared spheroids (Left; Mean values ± SEM for independently imaged spheroids; * indicates $p<0.05$ for two-tailed two-sample t-test between cleared and uncleared means).

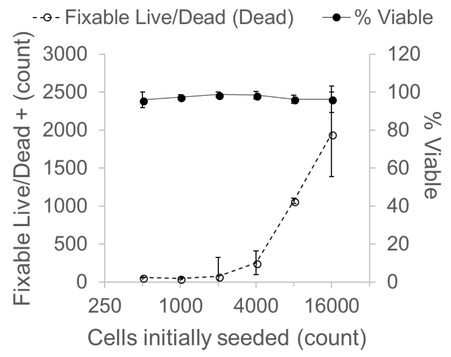


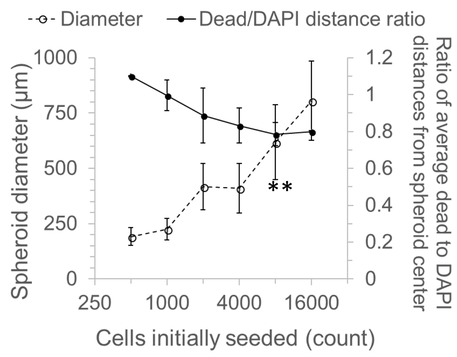


Results (cont.)

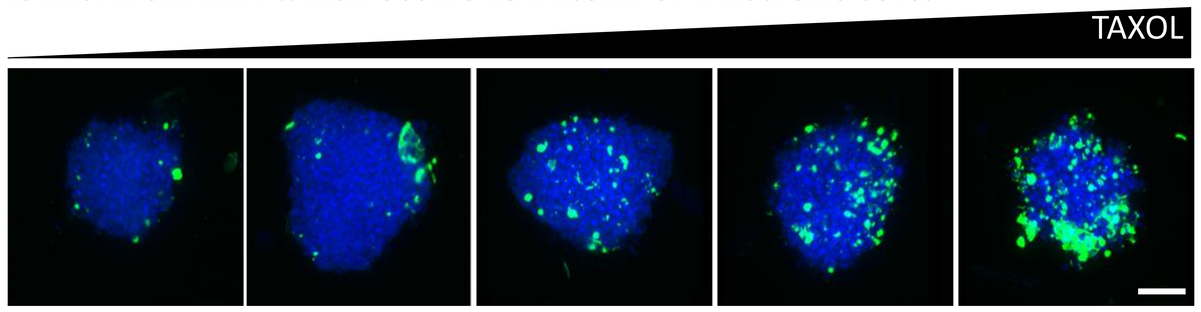
Cell seeding number is proportional to spheroid size and non-viable cell frequency, which corresponds with the development of a necrotic core

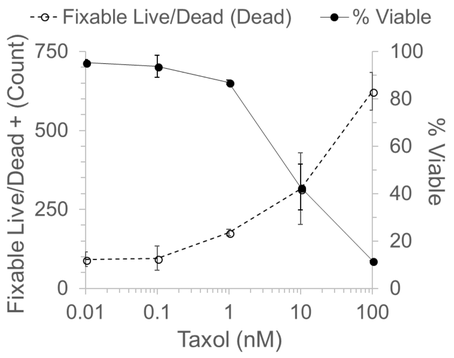
As more HepG2 cells are seeded, there is an increase in both the frequency of total and non-viable (top, dotted line) cells detected. Accordingly, the spheroid diameter increases (bottom, dotted line). However, even though the viability percentage (top, solid line) remains unchanged with increasing spheroid size, the distribution of non-viable cells suggests the formation of a necrotic core. More specifically, the ratio of the average distances of non-viable cells relative to that of all cells is expected to be ~1 if non-viable cells are uniformly distributed throughout the spheroid. However, larger spheroids (>300 µm) exhibited distance ratios <1, suggesting the non-viable cells are located closer to the spheroid center (bottom, solid line). Data represent mean ± SEM of independently imaged spheroids; ** indicates $p<0.01$ for multiplicity adjusted one-sample t-tests, H_o: µ=1.

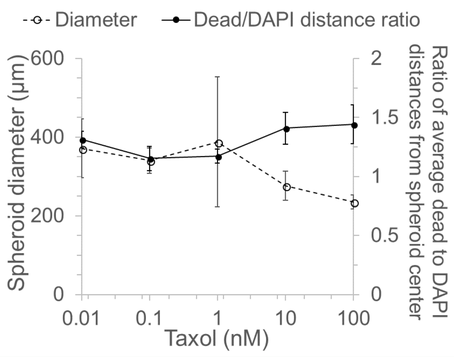




Treatment of HepG2 spheroids with taxol induces cell death in a pattern distinct from that which occurs as a result of a necrotic core.







Based on the appearance of a necrotic core in spheroids larger than ~300 µm in diameter, cells were seeded at a uniform density of 1,000 cells/well to achieve spheroids of ~250 µm in diameter. When treated with taxol, drug dose was directly proportional to the frequency of non-viable cells detected (above, scale bar 100 µm) and quantified (left top, dotted line). The percent viability correspondingly decreased with increasing taxol dose (left top, solid line), while the spheroid size remained relatively constant, except for highest treatment doses (10-100 nM), where some non-viable cells may have sloughed off of the spheroid (left bottom, dotted line).

Importantly, the ratio of average distances from the spheroid center of non-viable relative to total cells was >1 for all taxol doses assayed, suggesting the absence of a necrotic core. In fact, the ratio was found to be slightly larger than 1 in most cases, which is expected, given the drug concentration gradient likely to exist during at least a portion of the treatment duration.

Data represent mean values ± SEM for independently imaged spheroids; Multiplicity adjusted one-sample t-tests for ratio means revealed no means significantly <1.

Conclusions

- Visikol HISTO-M facilitates clear visualization of the spheroid interior, thus enabling the detection, quantification, and localization of non-viable cells.
- Spheroids larger than ~300 µm in diameter present a necrotic core with significant number of non-viable cells, a consideration that must be evaluated prior to implementing a 3D culture model.
- Detection and quantification of spheroid necrotic cores may prove crucial to the development of larger, more-complex multi-cellular spheroid models for high-throughput drug screening assays, where the mode of death- by therapeutic compound or by nature of the model- must be able to be distinguished.

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2. Gunness, P., Mueller, D., Shevchenko, V., et al. (2013) 3D Organotypic Cultures of Human HepaRG Cells: A Tool for In Vitro Toxicity Studies. Toxicolocial Sciences, 133(1), 67-78.
3. Nzou, G., Wicks, R.T., Wicks, E.E., et al. (2018). Human Cortex Spheroid with a Functional Blood Brain Barrier for High-Throughput Neurotoxicity Screening and Disease Modeling. Scientific Reports, 8(1), 7413.