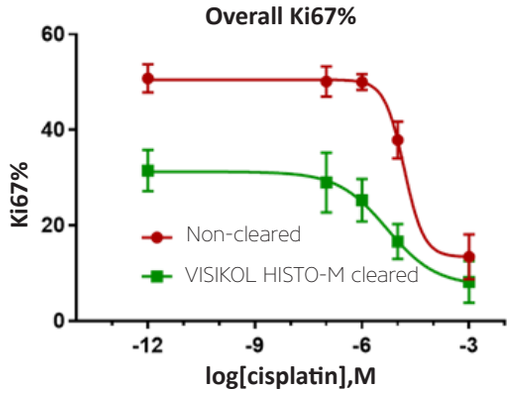

Application Note

3D high content imaging of optically cleared microtissues for cancer drug screening

When combined with high-content confocal microscopy, Visikol HISTO-M allows the interior environment of 3D cell cultures to be imaged, dramatically improving the number of cells detected in microtissues.

Introduction

The use of *in vitro* three-dimensional (3D) cell cultures for drug discovery has increased dramatically because 3D cell culture models more accurately mimic the *in vivo* environment compared to traditional monolayer cultures[1]. However, current imaging-based analysis of these 3D models relies upon techniques developed originally for 2D cell culture. Due to the thickness of 3D cell models, typically >100 µm, light scattering does not permit imaging of the center of the spheroid[2]. This technical limitation introduces a sampling bias in imaging analysis, since only the exterior cells can be imaged. This problem should be solved to obtain the most useful and accurate survey of the cellular environment and response of the microtissue.

It was sought to solve the problem of spheroid opacity by employing an optical clearing agent designed specifically for spheroids: Visikol HISTO-M. Here, we combine 3D InSight™ NCI-H2170 microtissues with Visikol® HISTO-M™ tissue clearing to illustrate the power of tissue clearing in a high content screening application.

Key Features

- > 3-fold increase in detectable cells within 3D cell cultures
- Designed for high-throughput applications and automation

Materials and Methods

Reagents and cell culture

NCI-H2170 lung cancer microtissues, co-cultured with normal human dermal fibroblasts, were obtained from InSphero Inc. (Cat # MT-01-028-03). Antibodies and fluorescent dyes were obtained from Invitrogen. Cisplatin was obtained from Sigma Aldrich.

Treatment of microtissues with cisplatin

Microtissues were treated with antiproliferative compound (cisplatin) on day 0, and again on days 3 and 6. Cisplatin 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

range of concentrations was 1 mM, 10 µM, 1 µM, 10 nM, 100 nM, and vehicle control.

Fixation and immunolabeling

On day 8, microtissues were fixed using 10% NBF, followed by washing in PBS to remove fixative. Microtissues were treated with methanol, followed by 20% DMSO/ methanol to improve penetration of antibodies and stains. Microtissues were blocked with 10% donkey serum, then incubated with rabbit anti-Ki67 antibody (1:1250 dilution, Abcam Cat# 15580) and with goat anti-rabbit AlexaFluor594 conjugated secondary (1:250 dilution, ThermoFisher Cat# r37117) to label proliferating cells. Nuclei were stained with SYTOX green (ThermoFisher).

Clearing and high throughput imaging of microtissues

Microtissues were dehydrated with methanol, then cleared with Visikol HISTO-M. Imaging of microtissue plates was accomplished with the CX7 (ThermoFisher) High Content Confocal Imager. Z-stacks were collected for each tissue, using 10 µm steps. Images were processed using automated ImageJ macros, and cells were counted using CellProfiler.

Results and Discussion

Visikol HISTO-M enables visualization of microtissue interior

As seen in Figure 1A, when imaging non-cleared 3D tissue cultures, the interior of the microtissue appears dark, as light scattering drastically reduces signal due to the opacity of the microtissues, causing the "eclipsing" effect. Using Visikol HISTO-M, light scattering was greatly reduced, allowing for comprehensive profiling of the interior of the microtissues, shown in Figure 1B. Note that Z-projections of non-cleared microtissues obscures the eclipsing effect that occurs due to tissue opacity and can result in misleading data.

Visikol HISTO-M increases detectable cells in interior

CellProfiler was used for automated cell-counting of the confocal image stacks. The detected cell outlines, shown in Figure 2A, show that as the image stack progresses into the non-cleared microtissue, fewer and fewer cells are detected in the center of each plane, until only the periphery is detectable. This is due to light scattering caused by the opacity of non-cleared microtissues, which limits imaging to approximately 20-50 µm depth, even with confocal microscopy. With cleared microtissues,

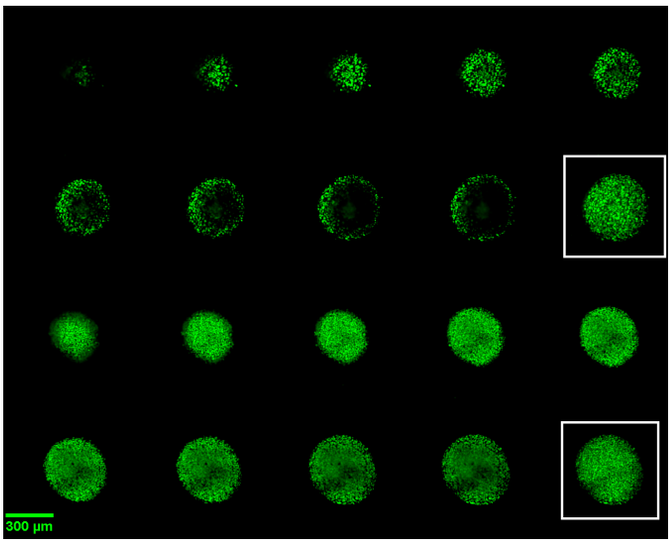

Figure 1.*Montage of slices from confocal image z-stack, nuclei stained with SYTOX green; A) Non-cleared spheroid; B) Cleared spheroid; Z-projections are outlined in white; z-projections mask difficulty of imaging non-cleared spheroids since z-projections hide the eclipsing that occurs in imaging. Cleared spheroids show no eclipsing, interior cells are visible.*

as shown in Figure 2B, cells are detectable across the entire image plane, deep into the microtissue. Cell counts for Figures 2A and 2B are quantified in Figure 2C. On average, 3-fold more cells were detected on each plane of the cleared microtissue than the non-cleared microtissue. The effect was even more dramatic deeper in the spheroid; at 120 µm, 7-fold more cells were detectable.

When conducting confocal imaging of microtissues without clearing, only the outermost cells are detected due to light scattering, which reduces signal to noise. Use of a tissue clearing agent greatly increases the number of cells detectable by high content imaging. The non-adherent surface coating of InSphero assay plates is chemically compatible with Visikol HISTO-M clearing. The clearing process takes only minutes, and is done directly in the 96-well plate.

Case study: Antiproliferative assay on NCI-H2170 microtissues

Cisplatin treated and control microtissues were imaged using a CX7 High Content Imager (ThermoFisher) to obtain multicolor image stacks with a 10 µm z-step size. Ki67 was used as a marker for proliferation. Cells were counted automatically with CellProfiler, giving total cell counts and Ki67+ cell counts. Ki67%, the ratio of Ki67+ to total cells, was used as the measurement of proliferation to construct dose response curves. Dose response curves were constructed for cleared and non-

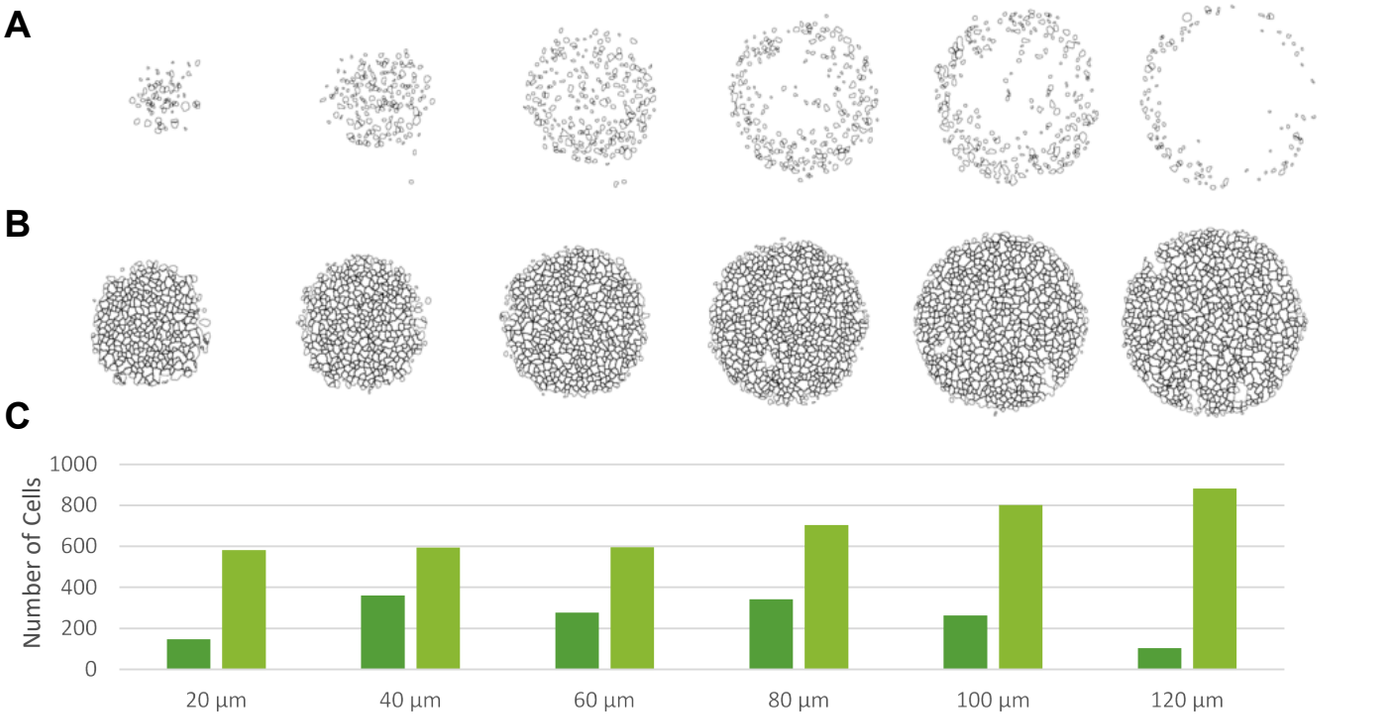

Figure 2. *Outlines of cells detected by CellProfiler in NCI-H2170 lung cancer spheroids treated with 10 nM cisplatin; 20 µm sections (20-120 µm) shown left to right for A) Non-cleared spheroids and B) Spheroids cleared with Visikol HISTO-M; C) Cell counts for each z-plane in cleared and non-cleared spheroids from A) and B). Far more cells can be detected at each z-plane in cleared spheroids.*

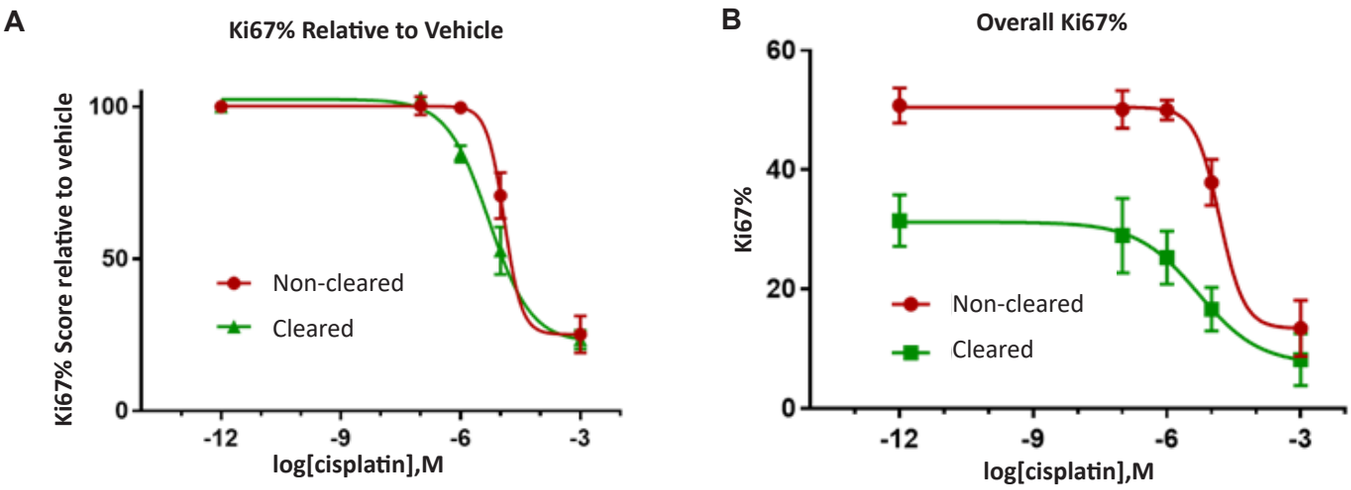

Figure 3. *Antiproliferation dose response curves for cisplatin-treated NCI-H2170 spheroids A) relative to vehicle control cell proliferation score; B) showing absolute Ki67% proliferation score.*

cleared microtissues, shown in Figure 3.

As can be easily discerned from the dose response curves shown in Figure 3A, there was a significant and measurable difference between dose response curves calculated from cleared and non-cleared microtissues. Visikol HISTO-M cleared samples show increased sensitivity to detect inhibitory activity (detected at 1 µM, compared to 10 µM required to detect signal for uncleared control samples). Furthermore, the IC_{50} value measured for Visikol HISTO-M (5.38 µM) is approximately 2x lower than calculated with non-cleared control samples (10.8 µM). These results were comparable with reported IC_{50} values. Visikol HISTO-M increases sensitivity to detect inhibition due to the increased number of cells surveyed compared to the non-cleared controls.

Another advantage of Visikol HISTO-M cleared microtissues is demonstrated by measuring the absolute reduction of proliferation score in cleared and non-cleared microtissues. As can be seen in Figure 3B, the cell proliferation score reduction for non-cleared

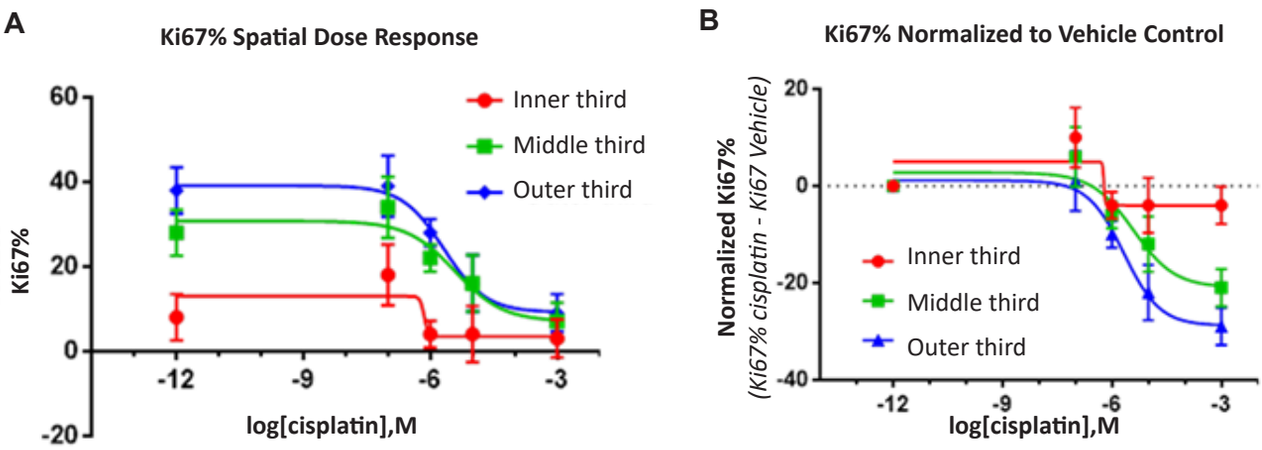

Figure 4. *Antiproliferation spatial dose response curves constructed using data obtained from cleared NCI-H2170 microtissues, splitting cells into innermost third, middle third, and outer third of cells to examine difference in response.*

tissues measures a greater reduction than for cleared tissues, which suggests that the non-cleared tissues give a falsely high rate of proliferation reduction. This is due to the bias toward surface-layer cells in imaging non-cleared tissues, which are more likely to be proliferating and likewise affected by cisplatin, and so shows a falsely overestimated response.

Spatial Dose Response

To assess the ability of a compound to penetrate and effect the deeper layers of cells, data from cleared microtissues can be further processed to measure dose response as a function of distance from the center of the spheroid (split into inner, middle, and outer thirds, Figure 4). A discernable difference in reduction of proliferating cells (Ki67% positive) by cisplatin is detectable between the inner third region, and both the middle and outer thirds. This effect is also evident when expressed as Ki67% change normalized to vehicle control.

Conclusions

3D tumor/stromal co-culture models are a valuable tool for studying the tumor microenvironment, as they more accurately reflect the complexities of ECM components, cellular arrangement, and resulting oxygen and nutrient gradients which impact cell growth and drug penetration. The combination of InSphero assay-ready microtissues and Visikol clearing reagent enables a more accurate, complete assessment of drug efficacy when using more physiologically relevant 3D models. Key findings include:

- Visikol HISTO-M is compatible with immunolabeling and fluorescent staining of microtissues
- Clearing microtissues with Visikol HISTO-M enables visualization of the interior of microtissues
- Cleared microtissues deliver > 3x higher cell counts with high-content confocal imaging assays
- Data obtained from Visikol HISTO-M better represents the microtissue since interior cells are detectable

References

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2. Kabadi, P. K., Vantangoli, M. M., Rodd, A. L., Leary, E., Madnick, S. J., Morgan, J. R., & Boekelheide, K. (2015). Into the depths: Techniques for *in vitro* three-dimensional microtissue visualization. Biotechniques, 59(5), 279.

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503-0004-01-A

InSphero AG
Schlieren, Switzerland
✆ +41 44 515 04 90

InSphero Inc.
Brunswick, ME USA
✆ +1 800-779-7558

Visikol, Inc.
New Brunswick, NJ U SA
✆ +1 800-615-8474

info@insphero.com
www.insphero.com

Authorized Distributors
www.insphero.com/distributors

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