High-Content Assays for Characterizing the Viability and Morphology of the 3D Cancer Spheroid Cultures

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INTRODUCTION

Three-dimensional (3D) in vitro models span the gap between two-dimensional cell cultures and whole-animal systems. By mimicking features of the in vivo environment and taking advantage of the same tools used to study cells in traditional cell culture, 3D models provide unique perspectives on the behavior of stem cells, developing tissues and organs, and tumors. These models may help to accelerate translational research in cancer biology and tissue engineering. High-content imaging is an emerging and efficient tool for generating multi-dimensional quantitative readouts in 3D cancer models.

Development of quantitative high-throughput in vitro assays which enable assessment of the viability and morphological changes is an active area of investigation. We optimized cell culture, staining, and imaging protocols for 96- and 384-well assay format and developed the workflow by designing a one-step procedure allowing reducing assay time and minimizing assay variability. We have also developed imaging and analysis methods providing multi-parametric characterization of the drug effects. The phenotypic readouts enabled by the improvement in the method include quantitative characterization of the size, shape of spheroids, as well as defining cell number, viability, cell proliferation and cell death within 3D spheroids.

To verify the robustness of the workflow, we tested a series of compounds that are established anti-cancer cytostatic and cytotoxic drugs. We demonstrate concentration-response effects of selected test compounds on HTC116 cell spheroids and illustrate how the proposed methods may be used for high-content high-throughput compound screening and evaluation of anti-cancer drugs.

GOAL: The goal of this study was to optimize cell culture and develop new imaging and analysis methods that can be used for compound screening through assessment of multiple phenotypes in human cancer 3D models.

MATERIALS & METHODS

- ImageXpress® Micro Confocal High-Content Imaging System – this model equipped with Widefield and Confocal (60µm pinhole) Optics
- MetaXpress® 6 High-Content Imaging Software
- Cells - HCT116 human colon cancer, DU145 human prostate cancer or HepG2 hepatocellular carcinoma (ATCC)
- Microplates – 96 or 384 well Corning U-shaped black wall, clear bottom plates (Corning 4520 and 3830, respectively)
- Hoechst 33342, Calcein AM, Ethidium Homodimer-1, CellEvent-Caspase 3/7, MitoTracker Orange CMTMRos (Life Technologies/Thermo Fisher)

ASSAY DEVELOPMENT

Spheroid Generation

We used low-attachment U-shaped black clear bottom plates to simplify cell culture, compound addition, and imaging. These plates eliminate spheroid transfer steps and center the spheroids in the wells, facilitating capture of an entire spheroid in one 10x or 20x image. Cells aggregate at the bottom of the U-shaped wells and form spheroids within 24 hours (Fig. 1). The thin plastic bottom of each well aids focusing and image acquisition with standard automated imaging systems. TL imaging was used for optimization. 1000-1500 cells/well were used for compound assays.

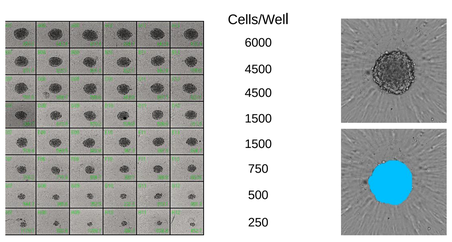
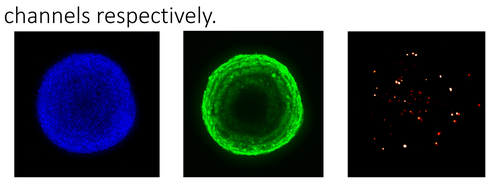


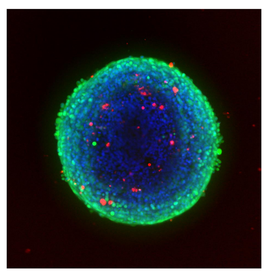
Figure 1. Image of spheroids generated in a 96 Well plate in transmitted light. Images analyzed using Custom Module Editor and spheroid areas measured.

RESULTS: IMPROVED ASSAY WORKFLOW

Staining

A one-step dye mixture addition was used to eliminate the need for fixing cells or repeated washes. Calcein AM was used to measure metabolically active cells, viability, and a variety of morphological parameters. Hoechst was utilized to measure total cell count and nuclear shape. EthD-1 selectively penetrates cells with damaged outer membranes and was used to measure dead or necrotic cells. Hoechst 15µM, EthD-1 3µM, and calcein AM 1µM. Images of Hoechst, calcein AM, and EthD-1 were taken using DAPI, FITC and Texas Red channels respectively.





Imaging

Taking only one image with a fixed offset does not allow adequate comparison of spheroids of different size or shapes, making acquisition of images at multiple focal planes necessary. Imaging protocols were studied using Hoechst stained spheroids. A series of images was acquired at different planes along the focal axis (Z-stack) and combined into a maximum projection (MaxPro) image.

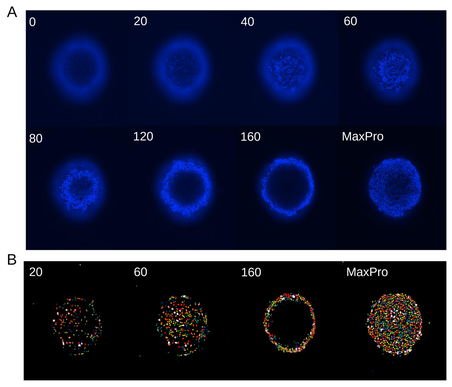
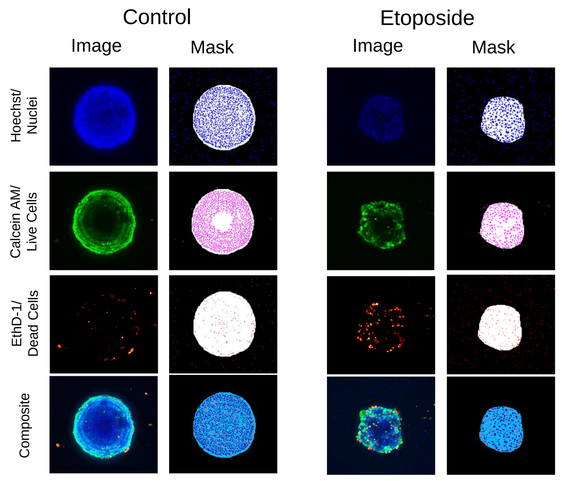
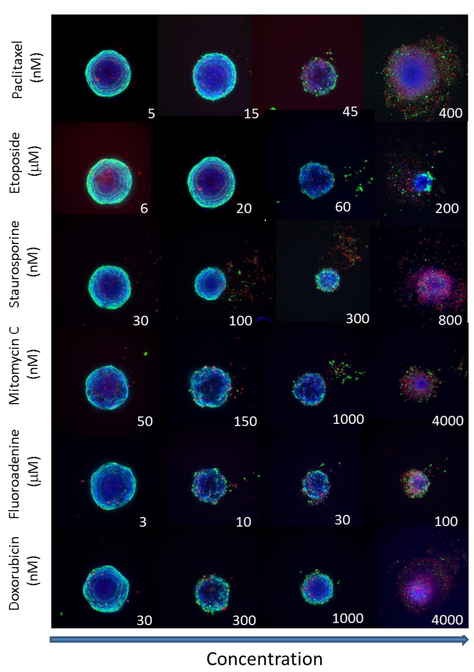


Figure 3. A. Confocal images of a spheroid stained with Hoechst taken at indicated distances from the well bottom. The MaxPro image is generated from 11 individual images taken 20 mm apart. B. Nuclei segmentation shown for corresponding images in A.

Phenotypic Analysis



SPHEROID PHENOTYPES



Spheroid assay performance was characterized using several compounds representing different classes of anti-cancer drugs: paclitaxel (microtubules formation), etoposide (topoisomerase inhibitor), staurosporine (kinase inhibitor), mitomycin C (DNA cross-linker), doxorubicin (anthracycline, DNA cross-linker), and fluoroadenine (purine analog). Complex phenotypes were observed.

Figure 4. images of spheroids treated with different compound concentrations. Dead cells appear red. Spheroids treated with high concentrations of some compounds (e.g., paclitaxel and staurosporine) appear to disintegrate.

Multi-Parametric Read-Outs and IC_{50} Values

Higher resolution spheroid imaging enables counting and scoring of individual cells. We counted the total number of cells in the image, the number of calcein AM-positive cells, the number of EthD-1 negative cells (Live), and the number of EthD-1- positive cells (Dead), as well as the average areas and intensities of cells expressing different markers.
MetaXpress CME used for multi-parametric image analysis to quantify different biological outputs.

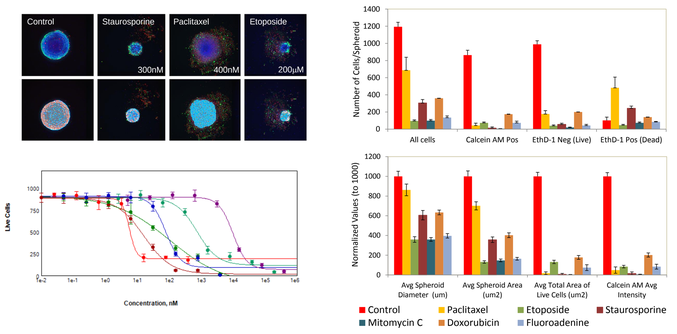
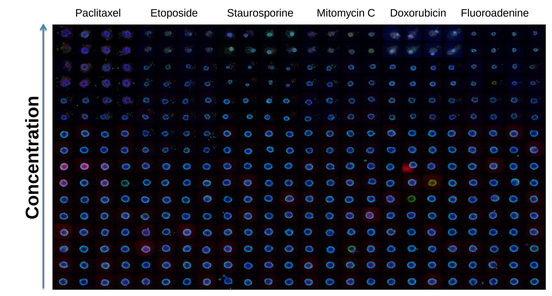


Figure 5. Image analysis read-outs derived as a result of Nuclei Count and Cell Scoring analysis : Bar graphs: control (0.1% DMSO), paclitaxel 150 nM, etoposide 200 µM, staurosporine 300 nM, mitomycin C 1µM, doxorubicin 1 µM, fluoroadenine 100 µM. Geometric or average intensity values in were normalized to DMSO controls (set to 1000). Concentration–dependent effects and 4-parameter curve fits of selected compounds. Red-paclitaxel, dark red-staurosporine, blue- doxorubicin, green- mitomycin C, teal- etoposide, purple- fluoroadenine.



COMPOUND LIBRARY SCREENING

To demonstrate utility of the assay we tested a library of 119 approved anti-cancer drugs and 20 control compounds across six concentrations from 0.1nM to 10µM. Of the test compounds, 74 caused a significant decrease (>3 SD from DMSO control) while 45 compounds showed no effect over the range of concentrations. The twenty negative controls also showed no effect at the tested concentrations.

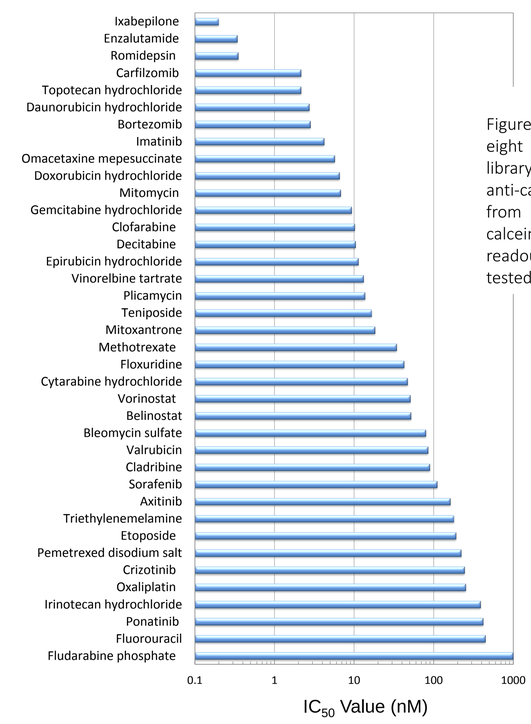


Figure 6. IC_{50} values of thirty-eight compounds from the library screen of 119 approved anti-cancer drugs obtained from 4-parameter fits to calcein AM-positive cells readout over the range of tested concentrations.

Summary

Using confocal system and high content imaging we have developed quantitative high-throughput assays which enable assessment of the viability and morphological changes in 3D cancer models.

Higher resolution and multi-parametric analysis allows single cell counting and classification to statistically characterize various spheroid phenotypes and drug effects.

The usefulness of the method for screening in 384-well format was demonstrated with a panel of anti-cancer drugs.

MOLECULAR DEVICES