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

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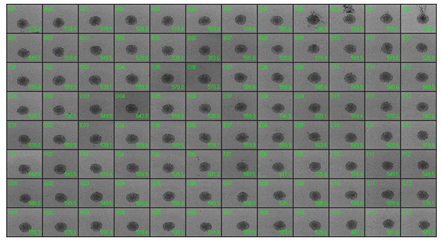
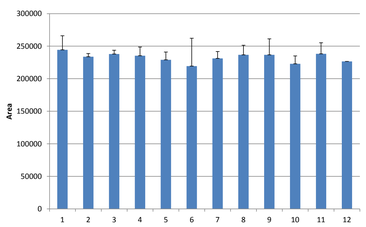
OVERVIEW

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. 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. Specifically, 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 protocols 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.

SPHEROID GENERATION

- HTC116 cells (ATCC) were plated into U-shape black clear bottom 96 or 384 well plates (Corning) and incubated in regular media for 48 hr.
- Spheroids treated with anti-cancer compounds for 5-7 days then stained using Hoechst (nuclei), Calcein AM (live cells) and Ethidium Homodimer (EthD, dead cells).





ImageXpress Micro XLS Imaging System

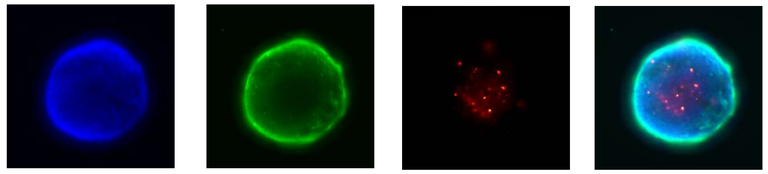
Figure 1. Top: Image of spheroids generated in a 96 well plate in transmitted light using ImageXpress Micro XLS system. Images were analyzed using MetaXpress Custom Module Editor to determine spheroid areas. Bottom: Average of measured of spheroid areas across each row. Each bar is an average of 8 spheroids and error bars represent 1 stdev. CV's of area measurements are ~ 10%.

MATERIALS & METHODS

- HCT 116 cells were received from ATCC and cultured in ultra low attachment U-shaped black clear bottom plates (Corning Spheroid Microplates, p/n 4520).
- Cells were treated with indicated compounds for 5 days (all compounds from Sigma Co.)
- Spheroids were stained with Calcein AM, Hoechst, and EthD (all from Life Technologies)
- Images were acquired with ImageXpress® Micro XLS automated imaging system using a 10x Objective and DAPI, FITC, & Texas Red filter cubes
- Images were acquired using Z-stack, 5-7 steps, then images were processed into a single 2D image "best focus" projection
- Images were analyzed using MetaXpress® software with the Custom Module Editor (CME).

ASSAY WORKFLOW - STAINING

Spheroids were stained with Hoechst, Calcein AM and Ethidium Homodimer to indicate presence of cells, cell viability, and membrane permeability. Staining protocols were optimized for uniformity throughout the spheroid. Analysis is possible in individual channels or using combinations for multi-parametric results.



Hoechst – Nuclei | CalceinAM – Viability | EthD – Dead Cells | Composite Image

SPHEROID PHENOTYPES

Distinct phenotypes are observed with different compounds. Compounds such as staurosporine can cause dramatic reduction of spheroid size at low concentrations, and cell death and disintegration of spheroids at high concentration. Mitomycin C leads to inhibition of growth and cell death. Etoposide mostly leads to shrinkage of spheroids. Image analysis methods have been developed to can quantify parameters that differentiate the phenotypes and allow concentration response profiling.

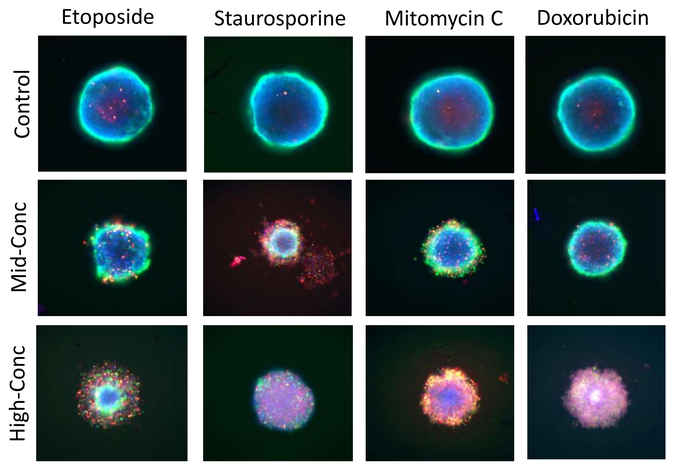
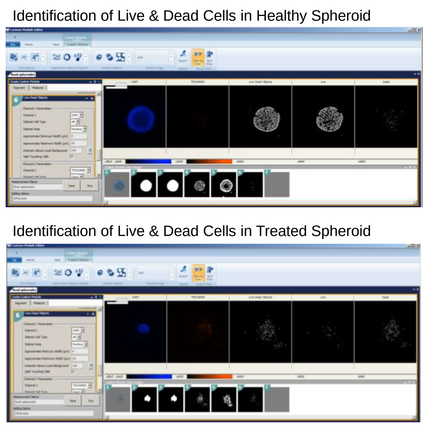
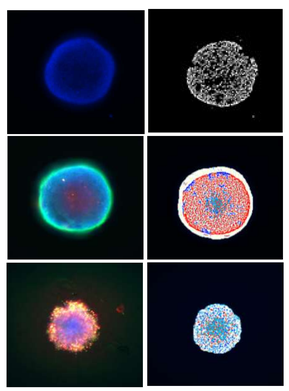


IMAGE ANALYSIS METHOD



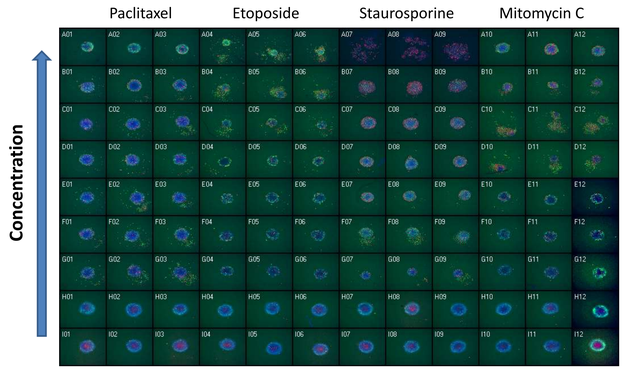


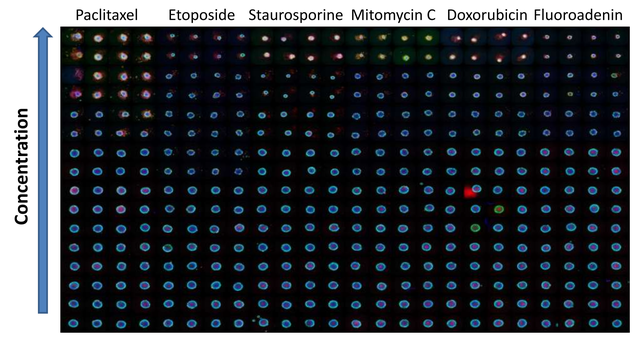
Red - Live Blue - Dead

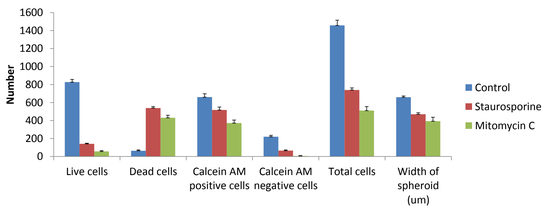
Figure 3. Custom Module was developed to 1) identify and segment spheroids from background, 2) segment cells within a spheroid, and 3) classify cells as live (unstained with EthD), dead (stained with EthD), positive or negative for Calcein AM. Analysis also allowed to define average intensities and areas of cells stained with different markers and also define width and area of projection.

COMPOUND TOXICITY RESULTS

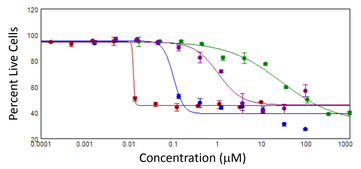
3D cultures are believed to allow more accurate prediction of the potency of anti-cancer drugs. Spheroids can be cultured efficiently in both 96 and 384 well plates allowing for higher through put evaluation of compound concentration responses.







IC50s	live cells	% live
Paclitaxel	0.013	0.013
Etoposide	2.3	28.4
Staurosporine	0.147	0.1
Mitomycin C	0.17	1.05



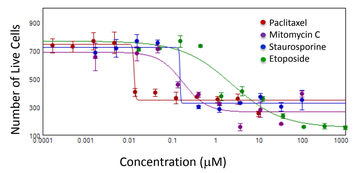


Figure 5. Top: Composite images of spheroid experiments in 96 and 384 well formats. Middle: Average output parameters for control and two compounds from 384 well experiment. Error bars represent 1 stdev (N=4). Bottom: Concentration responses measured for select compounds comparing Percent Live Cells and Total Live Cells. IC_{50} values are given the in table.

Summary

- Spheroids can be generated in U-shaped bottom wells suitable for imaging with excellent reproducibility.
- Protocols for multiple staining have been developed. Spheroid phenotypes can be elucidated using image analysis with MetaXpress software CME and multiple measurements.
- Different measurements provide expected dose-dependent responses to known compounds and can be used to compare compound efficacy.

MOLECULAR DEVICES