Morphometric Characterization of Cancer Spheroid Cultures Using Confocal Imaging and 3D Image Analysis

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INTRODUCTION

There is an emerging interest in using three-dimensional (3D) organoid cultures for modeling tissue biology and cancer. Development of higher throughput assays to quantify phenotypic changes in 3D models is an active area of investigation. The goal of this study was to develop higher-throughput, high-content imaging and analysis methods to characterize phenotypic changes in human cancer spheroids in response to compound treatment. We optimized spheroid cell culture protocols using low-adhesion U-bottom plates or solid media for three common cancer cell lines, and developed the workflow with a one-step staining procedure that minimizes variability. We used confocal imaging acquisition to characterize cellular information from multiple slices through a 3D matrix and objects, enabling efficient comparison of different spheroid phenotypes. 2D and 3D image analysis methods were implemented to provide multi-parametric characterization of single cell and spheroid phenotypes. We report a number of readouts including characterization of the number, size and shape or organoids, quantification of total or marker-specific cell numbers, as well as measurement of cell viability and apoptosis. We demonstrated concentration-response effects for different read-outs and measured IC_{50} values for the number of established anti-cancer cytostatic and cytotoxic drugs. The proposed methods can increase performance and throughput of high-content assays with 3D cell models for compound screening and evaluation of anti-cancer drugs.

GOAL: The goal of this study was to optimize the workflow 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
  - Equipped with Widefield and Confocal (60µm pinhole) Optics
- MetaXpress® 6 High-Content Imaging Software

ASSAY DEVELOPMENT

- 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)

Spheroid Generation

We used 2 different assay formats:

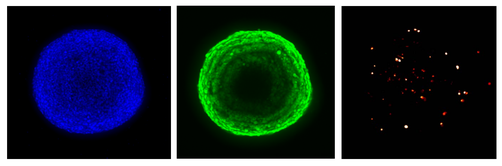
1. Single spheroid per well formed in low-attachment U-shaped black clear bottom plates (1000 cells/well). These plates eliminate spheroid transfer steps and center the spheroids in the wells, facilitating capture of an entire spheroid in one 10x or 20x image.
2. Multiple spheroids were grown in solid media (GF depleted Matrigel matrix). 400 cells were seeded into ½ well 96 plates.

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. Dyes concentrations: 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. In the alternative protocol cells were fixed using 4% formaldehyde, permeabilised with 0.02% saponin and stained with Hoechst and AF488-conjugated Phalloidin.

RESULTS: Phenotypic Analysis Using 2D Projection 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 typically combined into a maximum projection (MaxPro) image.



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 Custom Module used for multi-parametric image analysis to quantify different biological outputs. Spheroid assay performance was characterized using several compounds representing different classes of anti-cancer drugs.

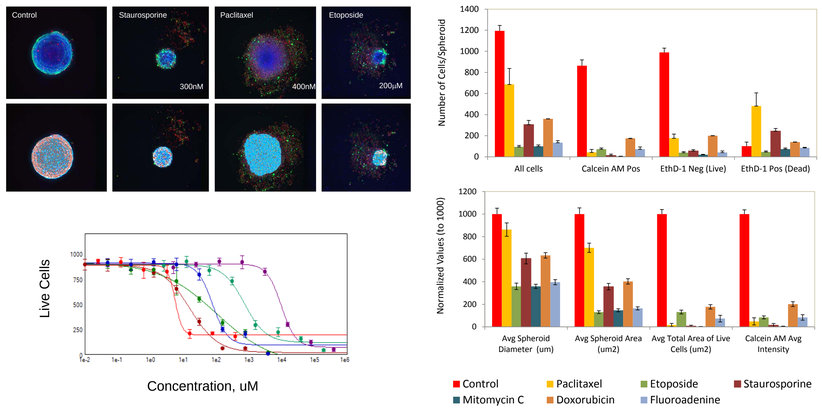


Figure 1. Maximum projection images of spheroids representing various phenotypes. Image analysis read-outs derived as a result of Nuclei Count and Cell Scoring analysis : Bar graphs: control (0.1% DMSO), paclitaxel 150 nM, etoposide 200 µM, staurosporine 300 nM, mitomycin C 1µM, doxorubicin 1 µM, fluoroadenine 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.

Analysis of Multiple Spheroids in Solid Media

Similar approach was taken for imaging and analysis of spheroids in semi-solid media. Images were acquired using confocal option, Z-slices were captured 5-10um apart, then maximum projections analyzed. Custom Module allowed count and characterization of individual spheroids, as well as count numbers of live and dead cells within spheroids. Compound treatment resulted in decreased numbers and size of colonies, also decrease of the numbers of live and total cells.

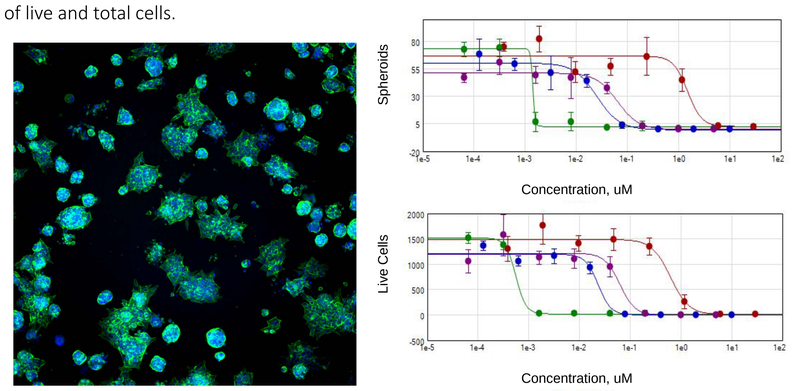


Figure 2. Maximum projection image of spheroids in Matrigel stained with Hoechst and AF488-phalloidin. Image analysis read-outs derived as a result of Custom Module including Cell Scoring analysis. Concentration–dependent effects and 4-parameter curve fits of selected compounds. Red-etoposide, green-paclitaxel, blue-staurosporine, purple- mitomycin C.

Characterization of Spherical Objects 3D Analysis

3D analysis enables the "link" of objects in different Z-planes, also characterization and morphometric analysis of spheroids or other objects in 3D. Spherical objects analysis allows accurate counting of all objects: spheroids or individual cells, as well as counting cells within spheroids. Multi-parametric image analysis was used to quantify different biological outputs.

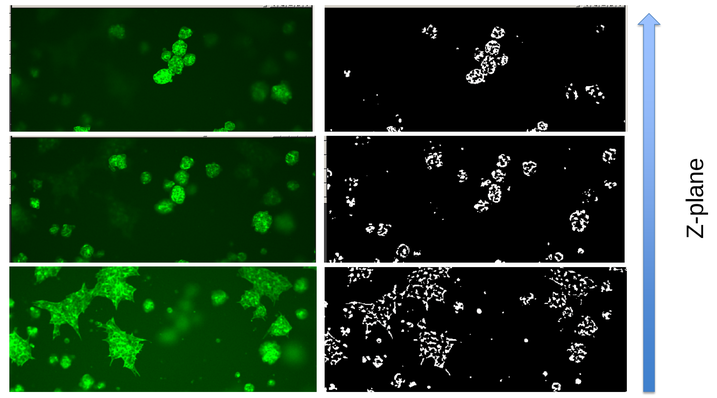


Figure 3. Individual Z-planes of spheroids in Matrigel stained with Hoechst and AF488-Phalloidin. Note changing of focus for different objects.

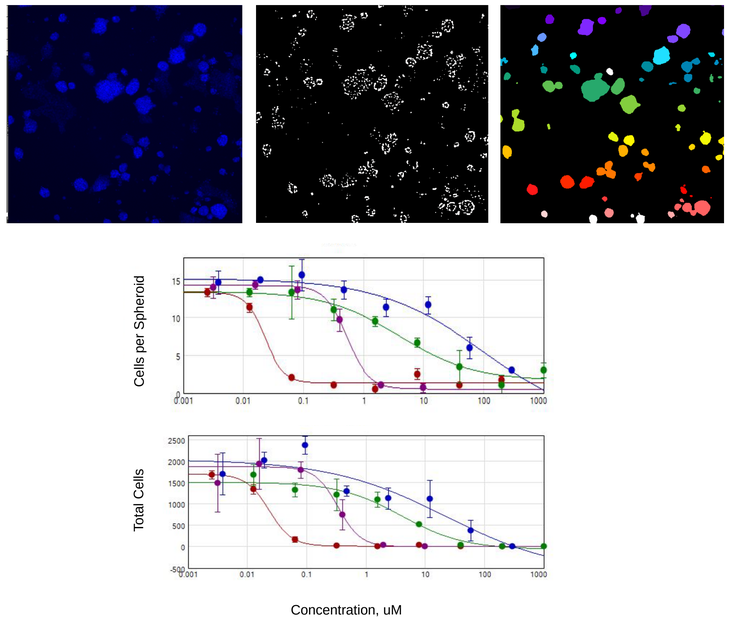


Figure 4. Count spheroids in Matrigel matrix as well as individual nuclei stained with Hoechst. Image analysis read-outs derived as a result of Custom Module including Spherical Objects analysis. Concentration–dependent effects and 4-parameter curve fits of selected compounds. Red-cytarabine, green-cisplatin, blue-fluoroadenin, purple-doxorubicin.

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.

2D and 3D analysis enables characterization of spheroids and individual cells and provide quantitative measurements that can be used for defining IC50s and comparison of potencies of various compounds. The usefulness of the method for screening in 384-well format was demonstrated with a panel of anti-cancer drugs.

MOLECULAR DEVICES