Image-based phenotypic profiling using Cell Painting in a 3D breast cancer spheroid model

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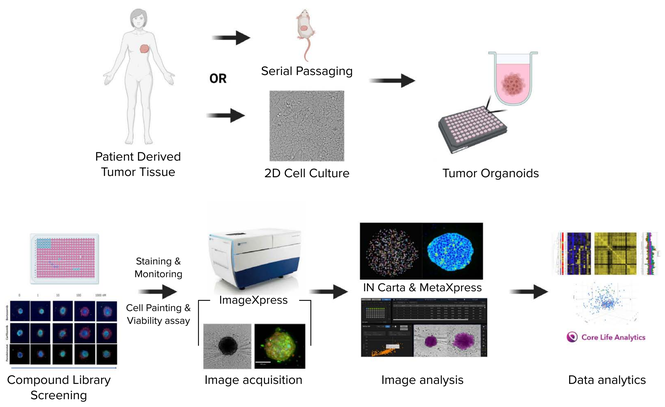
Introduction

Most potential oncology drugs fail the drug development pipeline, despite having promising data for their efficacy in vitro. This further incentivize the need for identifying in vitro models that better recapitulate tumor biology. Two-dimensional (2D) cell culture remains the primary method of drug screening, despite being less physiologically relevant than three-dimensional (3D) culture. In addition, challenges commonly associated with 3D cell models, such as assay reproducibility, scalability, and cost have limited its widespread adoption as a primary screening method in drug discovery. Moreover, the scope of biological readouts from 3D models is usually restricted to a single or a handful of features that do not fully capture the biological complexity of these tumoroids.

Image-based phenotypic profiling, such as with the Cell Painting assay, is increasingly used in many applications to quantitatively capture a broad range of phenotypic changes in response to compound-induced or genetic perturbations.

Here we performed a screen using patient-derived 3D spheroids (tumoroids). In addition to readouts on cell viability, we also adapted the Cell Painting assay for the 3D tumor model. Tumoroids were formed from primary cells isolated from a patient-derived tumor explant, TU-BcX-4IC, that represents metaplastic breast cancer with a triple-negative breast cancer subtype. Tumoroids were treated with 168 compounds from the NIH library of approved oncology drugs and Cell Painting was used to evaluate the associated phenotypic changes. We have additionally performed a single-feature readout from an image-based viability assay in parallel for comparison. Twenty-four hits were identified based on the phenotypic distance score that was calculated from the principal component analysis (PCA). Two-thirds of the hits overlapped with those from the image-based viability assay. Taken together, our results demonstrate the feasibility of using Cell Painting as an additional and important approach for 3D cell model analysis.



Schematic diagram of the experimental workflow including generation of a patient-derived cell line, formation of tumoroids in 384 well U-shape, low attachment plates, compound treatment, staining, imaging, and analysis.

Methods

Cell culture

The methods for generating tumoroids and PDX organoids (PDXO) have been previously described (Matossian, et al., 2021). The primary tumor sample was implanted into SCID/Beige mice and exhibited rapid tumor growth, with 14 days to reach maximal tumor volume >1000 mm^3. Then the cell line was generated from that sample and was expanded in 2D culture. Tumoroids were formed from 4IC cells expanded in 2D. 4IC cells were dispensed ~2,000 cells per well (in U-shape low attachment 384 plates [Corning]) and incubated for 48 hours until they formed tight tumoroids. 4IC cells were cultured with Advanced DMEM supplemented with glucose, NEAA, 2 mM glutamine and insulin 120 µg/L, 10% FBS (Gibco 12491-015). For metabolic assays, tumoroids were cultured with DMEM + 10% dialyzed serum (2 mM glutamine, 5mM glucose, without phenol red.

Spheroid monitoring and imaging

Transmitted light (TL) of fluorescent images were acquired on the ImageXpress® Confocal HT.ai High-Content Imaging System (Molecular Devices) using MetaXpress® High-Content Image Analysis Software. Tumoroid images were acquired in TL with approximately 60 µm offset. Z-stack images were acquired with the 10X or 20X objectives using confocal mode. MetaXpress or IN Carta® Image Analysis Software were used for analysis.

Cell Painting assay and data analysis

For the Cell Painting assay 17–20, the 3D TU-BcX-4IC tumoroids were labeled using a protocol modified from Bray et al. Tumoroids were incubated with MitoTrackerDeepRed (500 nM) (cat #) for 2 hours. The samples were fixed with 4% PFA in HBSS for 60 mins. All wash steps were carried out by exchanging half the volume in each well with HBSS to minimize displacement of the tumoroids from the center of the well. Following fixation, samples were washed three times with HBSS. For permeabilization, samples were incubated with 0.1% Triton X-100 (in HBSS) for 2 hr at room temperature and washed with HBSS. The dyes were prepared in HBSS and 1% BSA (wt/vol) and incubated overnight with the following final concentration: Hoechst (15 µg/ml), ConcanavalinA-488 (250 µg/ml), Syto14 (7.5 µM), Phalloidin750 (15 µl/ml), WGA (3.75 µg/ml).

Results

Spheroid culture and imaging

3D cancer culture was started from primary triple-negative tumor (see Methods section). The cell line was developed by passaging primary tissues in SCID mice, then adopted for 2D cell culture. Tumoroids were formed by culturing 2,000 cells in 384 well low-attachment plates for 48h, then tumoroids were treated with compounds from the National Cancer Institute (NCI) library of approved anti-cancer drugs. Five concentrations were used for testing (Figure 1).

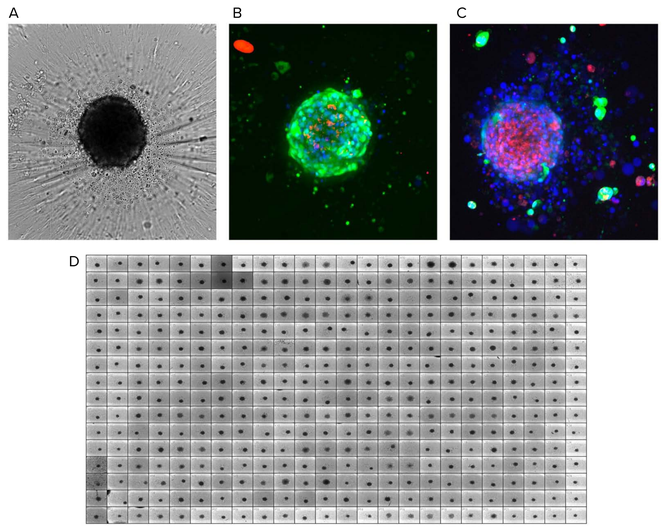


Figure 1. Reference images of tumoroids. A) Tumoroids formed 48h after plating, TL images (10X); B) Composite fluorescent images of untreated tumoroid stained with calcein AM (green), EthD-1 (red) and Hoechst (blue); C) Tumoroids treated with romidepsin (10 nM). Tumoroids were imaged using confocal option of the automated imaging system, Z-stack of 15 images was taken 10 µm apart, then maximum projection images were created (shown). D) 384 well plate with tumoroids after compound treatment, TL images.

High-content imaging and analysis of cancer spheroids

Tumoroids were treated with compounds at five concentrations (Figure 2) and then screened based on cell viability. In addition, phenotypic readouts that include tumoroid area, total cell count, and fluorescent intensity were quantified.

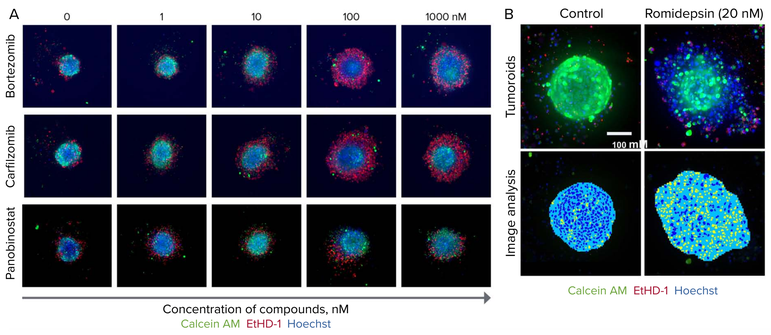


Figure 2. Tumoroids were treated with compounds for 5 days then stained with calcein AM (green), EthD-1 (red), and Hoechst (blue). A) Selected confocal images, 10X shown. Note dose-dependent dis-integration of tumoroids, increase in cell death indicated as increase of EthD-1 staining (in red). B) End-point analysis of fluorescent images was done using Custom Module Editor in MetaXpress. Images of the nuclei of treated and untreated tumoroid shown. Analysis masks show tumoroids projection in blue and nuclei in yellow. Tumoroid area and count nuclei were used as main read-outs for phenotypic characterization of tumoroids and compound effects.

Deep learning-based segmentation for label-free image analysis

Using IN Carta software, the image analysis routine can be adjusted to achieve robust detection of objects of interest (Figure 3). Deep-learning semantic segmentation module (SINAP) may be used to improve detection of challenging features. Here, a new model was developed to segment images of cancer spheroids acquired in transmitted light. Over 200 features (from all seven imaging channels) were subsequently derived from the spheroid segmentation mask.

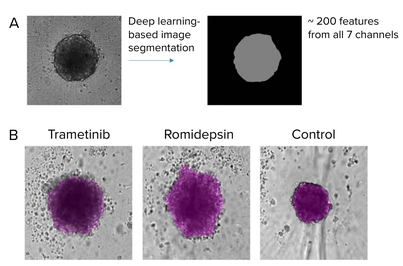


Figure 3. Automated image analysis of tumoroids was done using transmitted light images (label-free) (10X) with AI-based image analysis IN Carta software. A) SINAP (IN Carta) was used to develop a model to segment tumoroids. Over 200 features were then extracted from the segmentation mask from all 7 imaging channels (cell painting) that include stains for mitochondria, nuclei, ER, golgi, actin, RNAP and nucleoli. B) Examples of label-free images from tumoroids treated with different compounds and the corresponding segmentation mask (purple) from SINAP shown. Note that the SINAP model can identify tumoroids that are phenotypically different.

Results

Cell painting in phenotypic analysis of 3D spheroids

For a more in-depth investigation into cytotoxic mechanisms elicited by the compounds assayed, other analyses methods were performed in parallel to fully characterize phenotypic changes detected. The Cell Painting assay was adapted for 3D tumoroids for the evaluation of compound effects on tumor phenotype (Figure 4).

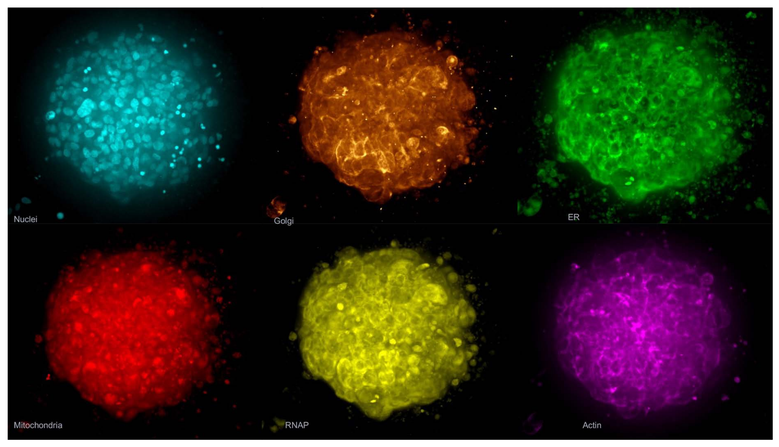


Figure 4. The Cell Painting assay modified for 3D spheroids. Spheroids were labeled with phalloidin, MitoTracker, WGA, SYTO 14, concanavalin A and Hoechst 33342. Shown here is an example image of a control spheroid (maximum projection) .

Data analysis workflow

Measurements from the IN Carta software were uploaded into HC StratoMineR for further data analysis. StratoMineR is a web-based platform which guides users through a typical workflow in analysis of high-content multi-parametric data (Figure 5).

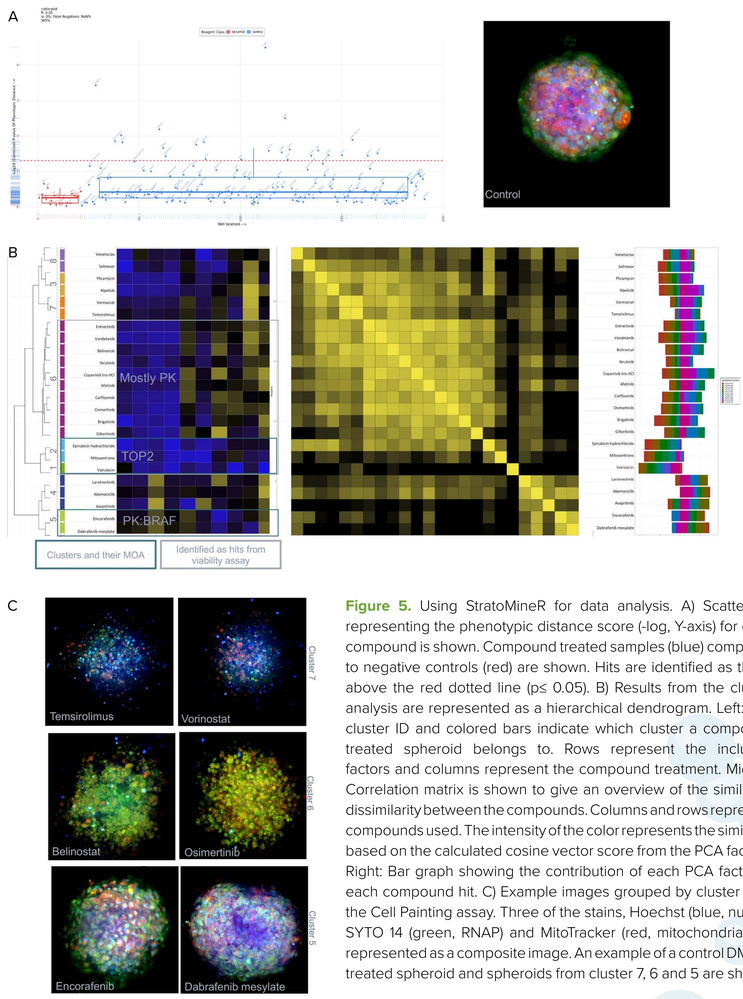


Figure 5. Using StratoMineR for data analysis. A) Scatterplot representing the phenotypic distance score (-log, Y-axis) for each compound is shown. Compound treated samples (blue) compared to negative controls (red) are shown. Hits are identified as those above the red dotted line (p≤ 0.05). B) Results from the cluster analysis are represented as a hierarchical dendrogram. Left: The cluster ID and colored bars indicate which cluster a compound treated spheroid belongs to. Rows represent the included factors and columns represent the compound treatment. Middle: Correlation matrix is shown to give an overview of the similarity/dissimilarity between the compounds. Columns and rows represent compounds used. The intensity of the color represents the similarity based on the calculated cosine vector score from the PCA factors. Right: Bar graph showing the contribution of each PCA factor to each compound hit. C) Example images grouped by cluster from the Cell Painting assay. Three of the stains, Hoechst (blue, nuclei), SYTO 14 (green, RNAP) and MitoTracker (red, mitochondria) are represented as a composite image. An example of a control DMSO-treated spheroid and spheroids from cluster 7, 6 and 5 are shown.

Conclusion

- Our results demonstrate the feasibility of using the Cell Painting assay on 3D cell models such as patient-derived tumoroids.
- Phenotypic profiling at the spheroid level was sufficient to identify compounds with cytotoxic and non-cytotoxic effects

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