# High-throughput chemotherapeutic drug screening of tumor spheroids with individual spheroid results using image cytometry

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## 1. OVERVIEW

We demonstrate an image cytometry assay that can kinetically monitor thousands of tumor spheroids individually and simultaneously. We collect size and fluorescence data from breast cancer spheroids and compare readings of tumor spheroid size to viability measurements obtained via an MTS assay.

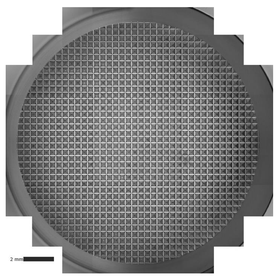
## 2. INTRODUCTION

Three-dimensional cancer models have gained popularity for *in vitro* studies of chemotherapeutic compounds by providing a more physiologically relevant analog of gas, nutrient, and drug diffusion throughout the tumor microenvironment. Some 3D assays are performed to study individual spheroids over time, where most of these assays rely on maintaining a single spheroid in each well of a 96-well round-bottom ultra-low attachment plate, limiting the number of spheroids in a study. Other assays may gather population-level data from large ensembles of spheroids grown together, but the information about individual differences amongst the spheroids is lost. Important kinetic information may also be lost for destructive endpoint assays such as MTS or MTT. Here, we describe the development of an image cytometry assay that can generate kinetic data for thousands of breast cancer spheroids at the individual level. T47D spheroids are grown and maintained in a 24-well Aggrewell™400 plate (STEMCELL Technologies) and imaged using the Celigo® image cytometer (Revvity). Each well contains more than 1000 subwells that both aid in spheroid formation and constrain each spheroid to a specific location. Using the spheroid location data, we are able to track and monitor the growth of each spheroid over time. Furthermore, we investigate the dose-dependent effects on spheroid viability of 6 anti-cancer drugs (Doxorubicin, Everolimus, Gemcitabine, Metformin, Paclitaxel and Tamoxifen) using calcein AM and propidium iodide (PI). To validate the results, we compare dose-dependent trends in spheroid diameter with viability readings obtained from the CellTiter96® MTS assay (Promega Corporation). This work may lay a foundation for the investigation of other spheroids, organoids, or tissue samples, significantly increasing the number of spheroids analyzed per condition, improving the statistical analysis, and adding more parameters to further analyze the spheroids. These improvements may be especially helpful for spheroids grown from patient-derived or otherwise heterogeneous cell populations.

## 3. SEEDING DENSITY

**Aggrewell™400 Plates:**

- Each well of an Aggrewell™400 plate contains about 1200 square subwells, 400 µm on each side.
- When a cell suspension is loaded into the wells, cells settle into clumps in the centers of the subwells.
- For certain cell lines, the clumps of cells will form compact spheroids.

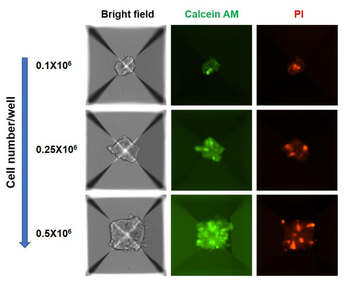


**Celigo® Imaging Cytometer:**

- The Celigo® is an automated plate-based imager designed for speed and minimal distortion at the edges of wells.
- The instrument can rapidly capture images in brightfield and up to 4 fluorescence channels (ex/em: 628/688, 531/629, 483/536, 377/470).
- For this study, we captured images in brightfield, 483/536 for calcein AM, and 531/629 for Propidium Iodide.

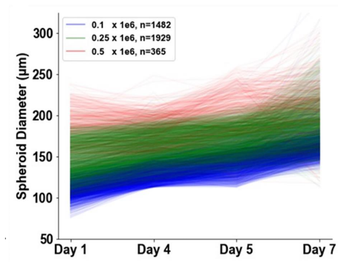

**T47D Breast Cancer Spheroid Formation:**

- We confirmed that increasing the cell seeding density increases the size of the spheroids, as expected.
- We stained the spheroids with calcein AM (green) and PI (dead) at increasing cell numbers seeded per well ranging from 0.1–0.5 × $10^6$ cells/well.
- These images were taken on day 1 post cell seeding.




**Individual Spheroid Tracking:**

- We performed image analysis using Celigo's built-in applications.
- Using exported data for each spheroid's location, we identified individual spheroids from scans spanning several days.



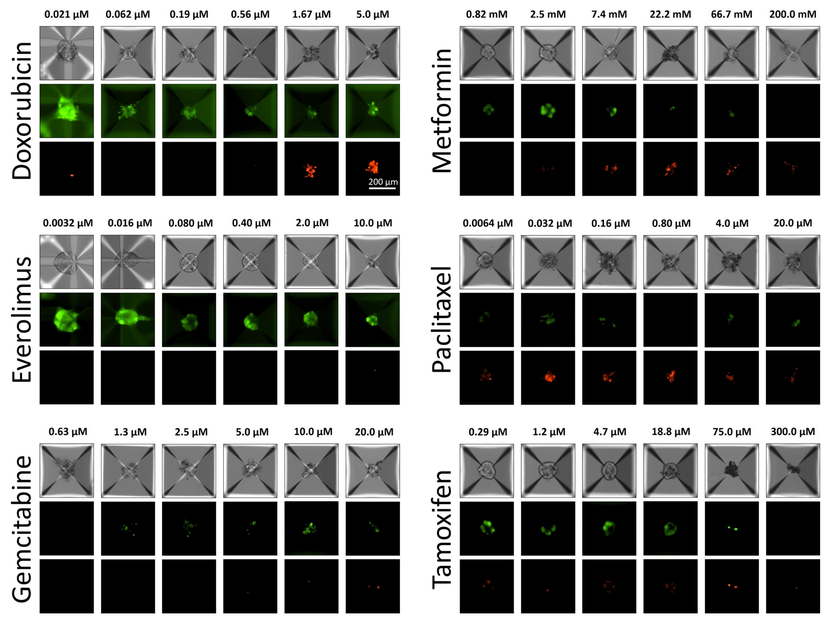

## 4. SPHEROIDS SUBJECTED TO 6 ANTI-CANCER DRUGS IN 6 DOSES

**Treatment**

- Doxorubicin 0.02 - 5 µM
- Metformin 0.82 - 200 mM
- Everolimus 0.0032 - 10 µM
- Paclitaxel 0.0064 - 20 µM
- Gemcitabine 0.0625 - 20 µM
- Tamoxifen 0.29 - 300 µM

**Imaging**

- Images were acquired on Day 7 after drug treatment.
- Spheroids were stained with calcein AM (shown in green) and Propidium Iodide (shown in red).
- Plates were imaged on the Celigo® imaging cytometer.




## 5. EFFECT OF ANTI-CANCER DRUGS ON CALCEIN AM AND PROPIDIUM IODIDE FLUORESCENCE INTENSITY

**Fluorescence Intensity vs. Drug Dose**

- Seven days after treatment, we used Celigo® to measure the mean fluorescence intensity for each spheroid for both calcein AM (live cells) and PI (dead cells).
- The mean intensity distribution for each drug/dose combination is represented by approximately 2400 spheroids (2 wells).
- Many of the conditions show statistically significant differences relative to the highest dose, but most of the effects are minor relative to the overall variation among the spheroids.



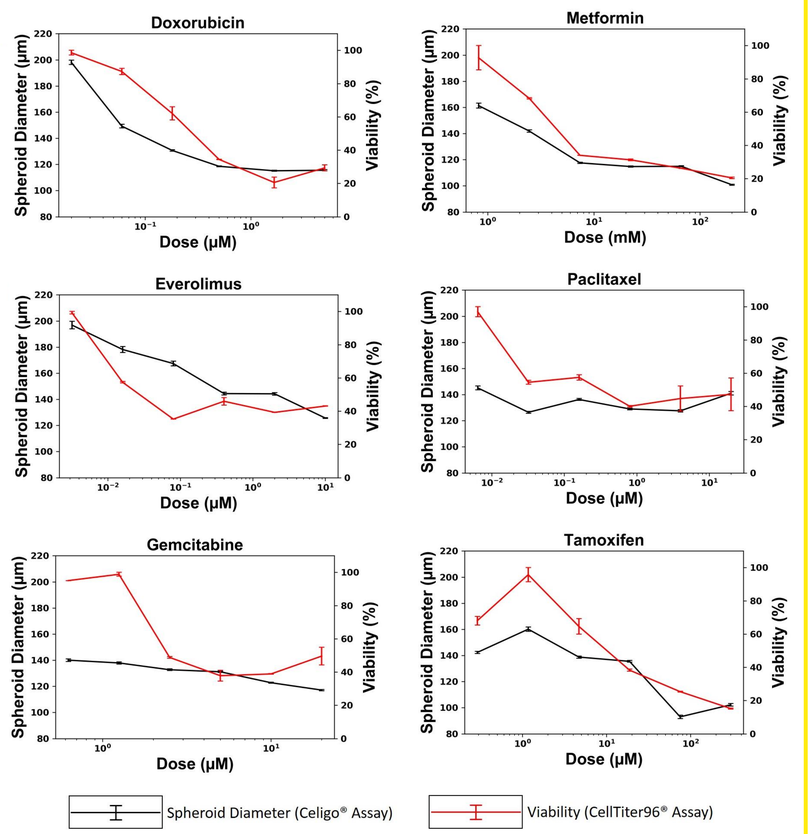

Statistical significance calculated against the highest dose of each drug using one-way ANOVA with Tukey post-hoc test

## 6. SIZE OF SPHEROIDS COMPARED TO MTS VIABILITY MEASUREMENT

**Spheroid Diameter vs. Drug Dose**

- We measured the spheroid sizes 7 days after drug treatment.
- Once imaging was complete, we measured the viability of the spheroids using the endpoint CellTiter96® MTS assay.
- Doxorubicin, Metformin, and Tamoxifen showed statistically significant correlation ($p < 0.05$) between spheroid diameter and viability.

| Treatment | Pearson Correlation (r) Between Diameter and Viability | P Value |
|---|---|---|
| Doxorubicin | 0.90 | 0.015 |
| Metformin | 0.99 | 0.00015 |
| Everolimus | 0.75 | 0.086 |
| Paclitaxel | 0.70 | 0.12 |
| Gemcitabine | 0.72 | 0.11 |
| Tamoxifen | 0.91 | 0.011 |




The data reported here are based on cumulative diameter and viability of approximately 1200 spheroids/well and 2 wells/condition for Doxorubicin, Everolimus, and Gemcitabine and 1 well/condition for Metformin, Paclitaxel, and Tamoxifen. The MTS assay was performed in duplicates. Error bars indicate standard error of the mean (SE).

## 7. CONCLUSIONS

- In combination with Aggrewell™400 plates, the Celigo® imaging cytometer can measure size and fluorescence intensity for thousands of tumor spheroids at once.
- Additional analysis of exported data allows spheroids to be tracked on an individual level.
- When performed with calcein AM and PI on T47D spheroids, the assay gave results comparable to the traditional MTS viability assay for a 6-drug panel.

## 8. READ THE FULL PAPER

"Automated Assessment of Cancer Drug Efficacy On Breast Tumor Spheroids in Aggrewell™400 Plates Using Image Cytometry"

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