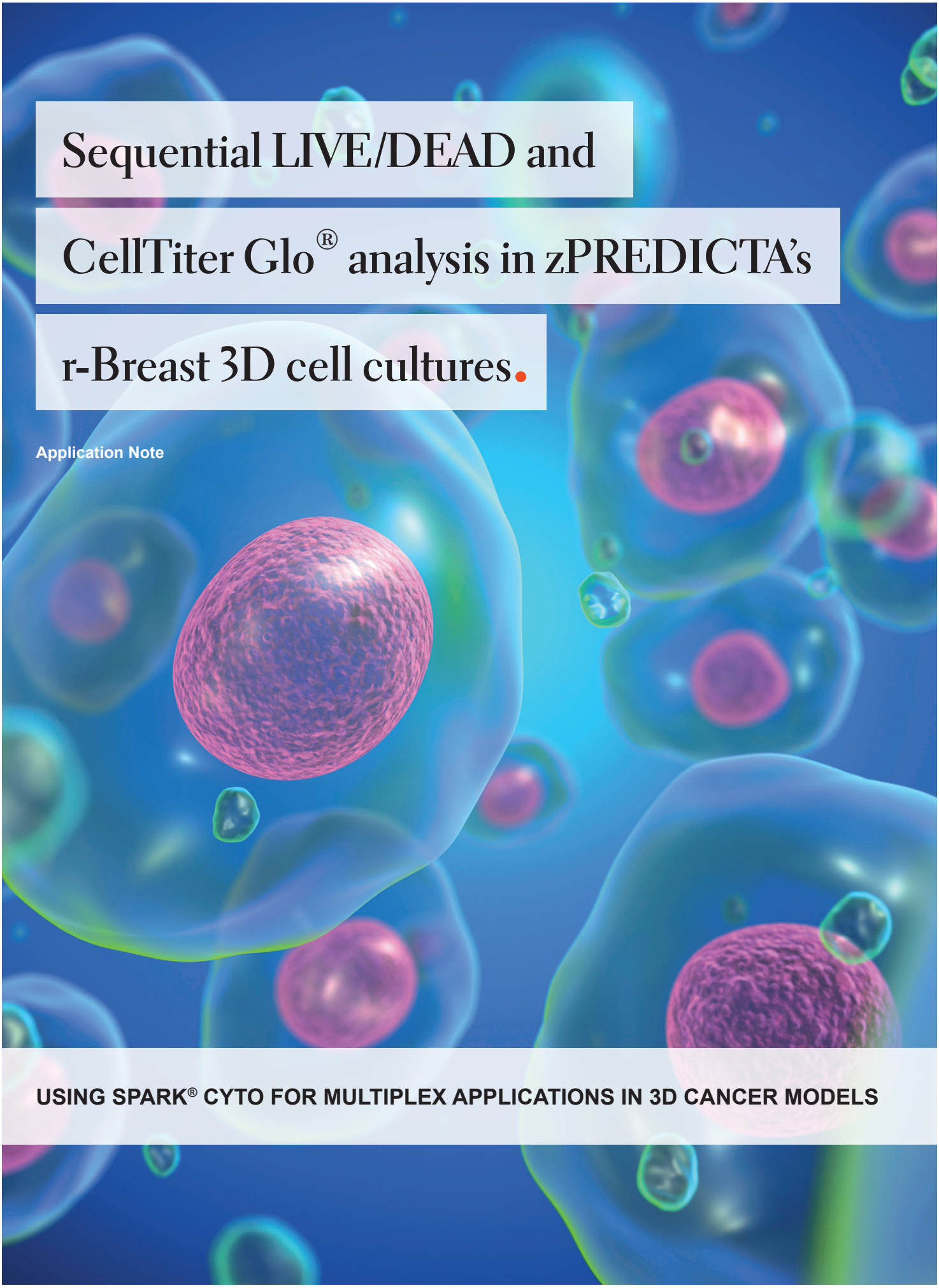
Sequential LIVE/DEAD and CellTiter Glo® analysis in zPREDICTA's r-Breast 3D cell cultures.

Application Note



USING SPARK® CYTO FOR MULTIPLEX APPLICATIONS IN 3D CANCER MODELS

INTRODUCTION

Standard cell culture methods – where cells grow as monolayers on the plastic surface of a flask or plate – may not accurately model cancer treatment responses, as they lack the complexity of the tumor microenvironment. This can influence how tumor cells respond to a drug or become resistant to treatment. For an anticancer drug to be effective in patients, it first needs to overcome the protection conferred by the interaction between tumor cells and the extracellular matrix (ECM), stromal and immune cells, and secreted factors. zPREDICTA's r-Breast and r-mBreast 3D culture models aim to reconstruct the microenvironment of human and mouse mammary tumors, respectively. They mimic the epithelial and stromal niches within mammary tissue, providing a more physiologically relevant tool for cancer researchers to evaluate novel drugs or drug combinations, capturing species-specific differences in ECM composition.

While 3D cultures are superior to the 2D culture approaches in terms of physiological tissue organization and providing robust prediction of clinical outcomes, these techniques are often more expensive and time-consuming to perform compared to the standard 2D protocols. Performing multiplexed assays with various readouts is therefore beneficial in order to gain the most information from each experiment. Here, we describe a multiplexed approach combining sequential imaging-based LIVE/DEAD cell viability assays (Thermo Fisher Scientific) and CellTiter Glo luminescence analysis (Promega) using the Tecan Spark Cyto with r-Breast and r-mBreast models.

r-Breast and r-mBreast culture set-up

The r-Breast/r-mBreast 3D culture set-up involves a simple, four step process (Figure 1). While the ECM compositions differ between human and murine systems, reflecting interspecies variation, the set-up process is the same for both models.

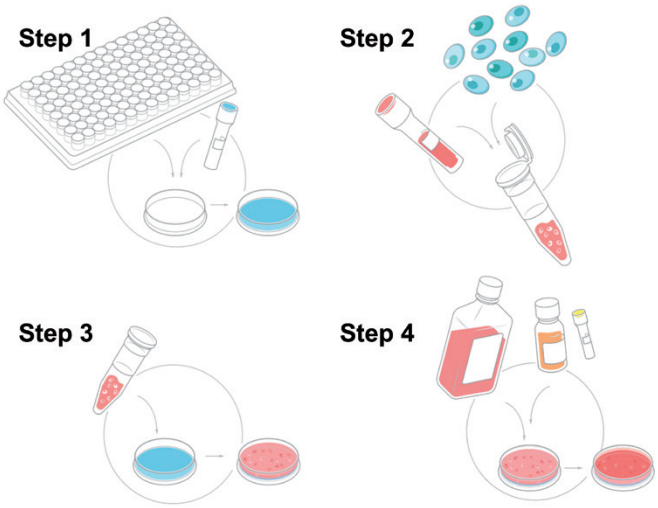


Figure 1: Four step set-up of r-Breast/r-mBreast 3D cultures. **1)** Coat plates with stromal ECM. **2)** Mix cells with r-Breast (or r-mBreast) ECM. **3)** Overlay stromal ECM with the cells/r-Breast ECM mixture, and polymerization. **4)** Add growth medium with disease-specific supplements.

In the first step of the protocol, tissue culture plates are coated with a matrix formulated to reconstruct the stromal compartment of the mammary tissue. Breast cancer cell lines are then mixed with the r-Breast epithelial ECM, layered over the stromal ECM on the plates, and allowed to polymerize, forming an 800 to 1,000 µm 3D layer. Finally, growth medium supplemented with tumor-specific soluble factors is added to the cultures. For a more complex tumor environment set-up, stromal cells or cancer-associated fibroblasts (CAFs) may be added to the 3D layer for epithelial/stromal co-cultures.

After 3 to 5 days, the breast cancer cells establish a complex tissue architecture consistent with their behavior *in vivo*. Some cell lines form spheroids, while others form stellate or network structures (Figure 2).

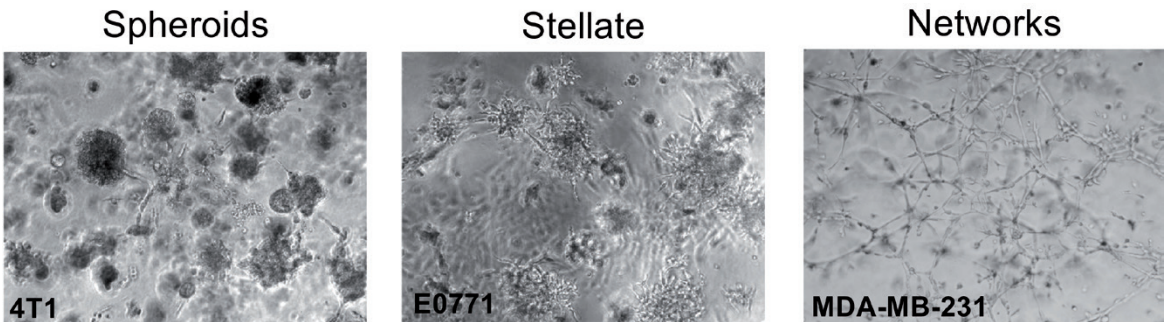


Figure 2: Typical 3D architecture formed by breast cancer cell lines using the r-Breast and r-mBreast platforms. Murine 4T1 and E0771 cell lines were cultured for 14 days in r-mBreast, forming spheroids and stellate structures, respectively. The human MDA-MB-231 cell line was cultured with r-Breast and formed networks. Bright field images were collected on the Spark Cyto.

Once the 3D cultures are established, cells can be treated with different therapeutic agents – such as small molecules, antibodies, antibody-drug conjugates, bi-/tri-specific agents or CAR-T cells – and responses can be evaluated *in situ* using microscopy, CellTiter Glo assays, immunohistochemistry, etc. The cells or spheroids can also be separated from the r-Breast ECM by non-enzymatic digestion for downstream applications, such as flow cytometry, NGS or *in vivo* transplantation.

LIVE/DEAD cell imaging in r-Breast 3D cultures using the Spark Cyto

One of the main advantages of the zPREDICTA's 3D culture platforms is that cells can be maintained in culture for long periods of time, allowing the study of both fast- and slow-acting drugs. The LIVE/DEAD assay is a robust technique that can be used to evaluate cell survival in complex culture architectures over time, as well as to test viability changes in response to anticancer treatments. To analyze cell viability in the r-Breast and r-mBreast cultures *in situ*, cells were simultaneously stained with green fluorescent calcein AM (to detect live cells) and red fluorescent ethidium homodimer-1 (to detect dead cells), according to the manufacturer's protocol (Figure 3).

In situ **LIVE/DEAD staining procedure (96-well plates):**

1. Remove culture media without disturbing the ECM matrix.
2. Add 100 µl/well of LIVE/DEAD staining solution (2 µM calcein AM and 4 µM ethidium homodimer in PBS buffer).
3. Incubate for 30 min at room temperature in the dark.
4. Image the cells within 1 hour of staining in the Spark Cyto with the green and red optical filter sets. For 3D cultures, set focus offset to 300-400 µm, and exposure time to 10-50 ms, using the 10X objective.

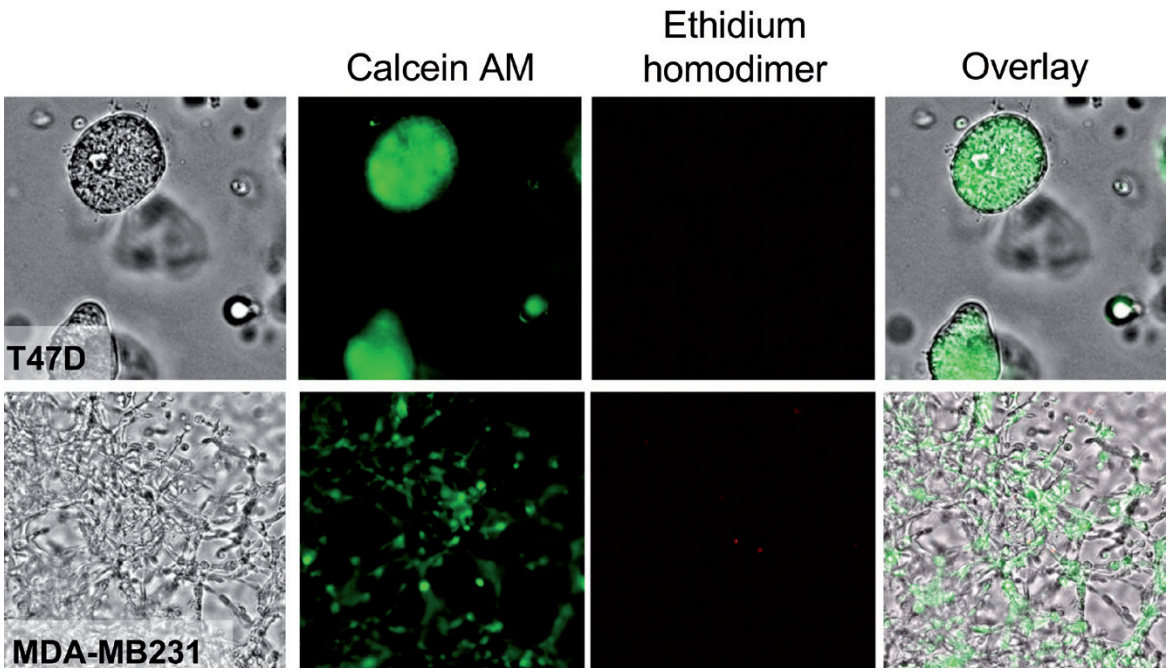


Figure 3: Viability analysis in r-Breast 3D cultures using a LIVE/DEAD *in situ* assay. Human MDA-MB-231 and T47D cell lines were cultured using the r-Breast platform for 14 days before staining and imaging. Bright field and fluorescence images were collected on the Spark Cyto.

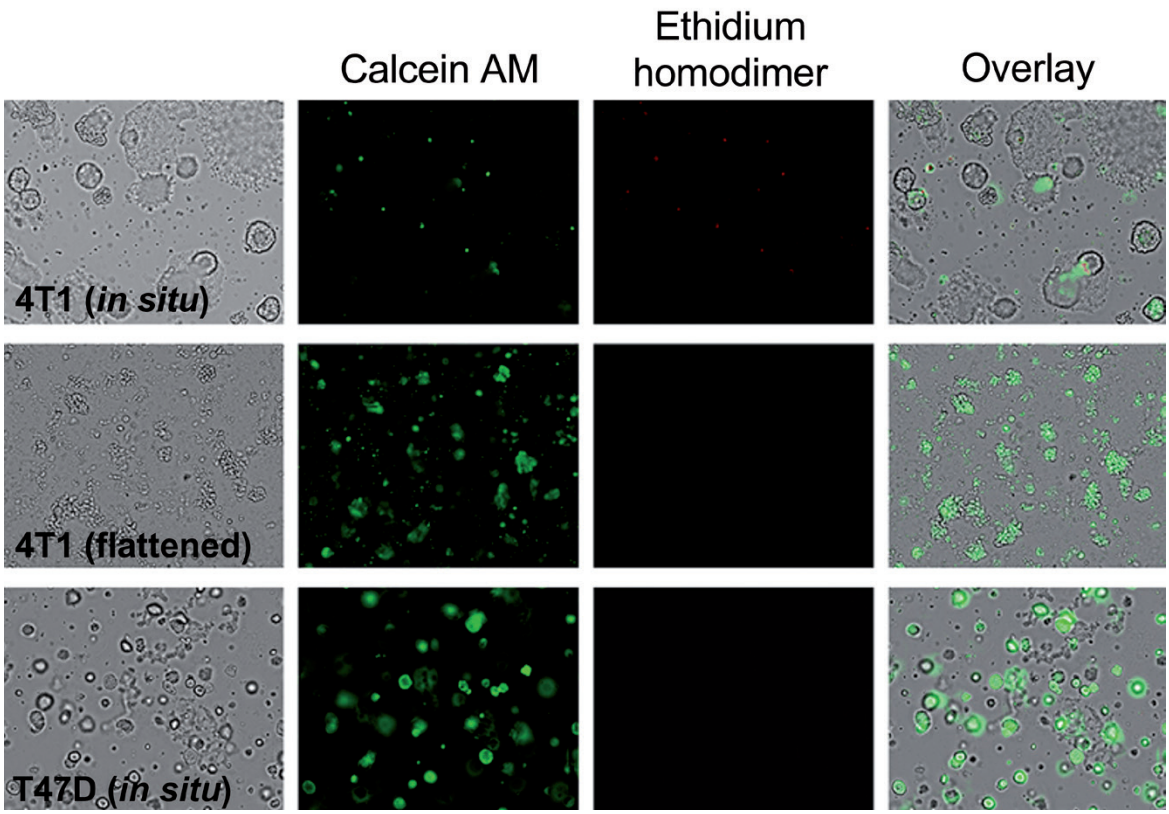


Figure 4: Viability analysis of cell lines with poor uptake of calcein AM. Murine 4T1 and human T47D breast cancer cell lines were cultured for 14 days in r-mBreast and r-Breast respectively. Cultures were subsequently tested for viability using a LIVE/DEAD assay, according to the *in situ* or the ECM depolymerization protocols. Phase contrast and fluorescence images were collected on the Spark Cyto.

Although most cells can efficiently incorporate LIVE/DEAD reagents, the murine 4T1 cell line failed to stain with calcein AM with any of the concentrations recommended in the protocol, independent of cell viability, which was confirmed using another method (data not shown). To overcome this, zPREDICTA's non-enzymatic cell isolation solution (CIS) was used to depolymerize the ECM and 'flatten' the cultures prior to staining with LIVE/DEAD reagents (Figure 4).

Matrix depolymerization procedure (96-well plates):

1. Remove 140 µl of media from each well without disturbing the ECM matrix, leaving ~60 µl of media in the well (assuming a total starting volume of 200 µl/well).
2. Add 200 µl cold CIS and gently aspirate and dispense 3-4 times to break up the ECM.
3. Incubate the plate at 4 oC for 1 h to allow the matrix to depolymerize. Spheroids and single cells will sink to the bottom of the well.
4. Carefully discard the supernatant without disturbing the cells.
5. Proceed with step 3 of the *in situ* LIVE/DEAD staining protocol.

CellTiter Glo analysis using the Spark Cyto

The CellTiter Glo assay is a widely-used, standard method for evaluating cell proliferation, establishing growth curves and measuring the cytotoxic or antiproliferative effects of certain anticancer drugs. This approach was used to demonstrate the potency of the chemotherapy drugs paclitaxel and cisplatin in the MDA-MB-231 (triple negative) and T47D estrogen receptor-positive/progesterone receptor-positive (ER+PR+) breast cancer cell lines grown in r-Breast (outlined below, results in Figure 5). Both cell lines were more sensitive to paclitaxel than to cisplatin treatment, as expected. Cells cultured using the r-Breast platform were more resistant to treatment with either compound compared to cells cultured under standard 2D conditions. This is likely due to the r-Breast ECM and media supplements more closely mimicking the tumor microenvironment, which confers some protection to tumor cells.

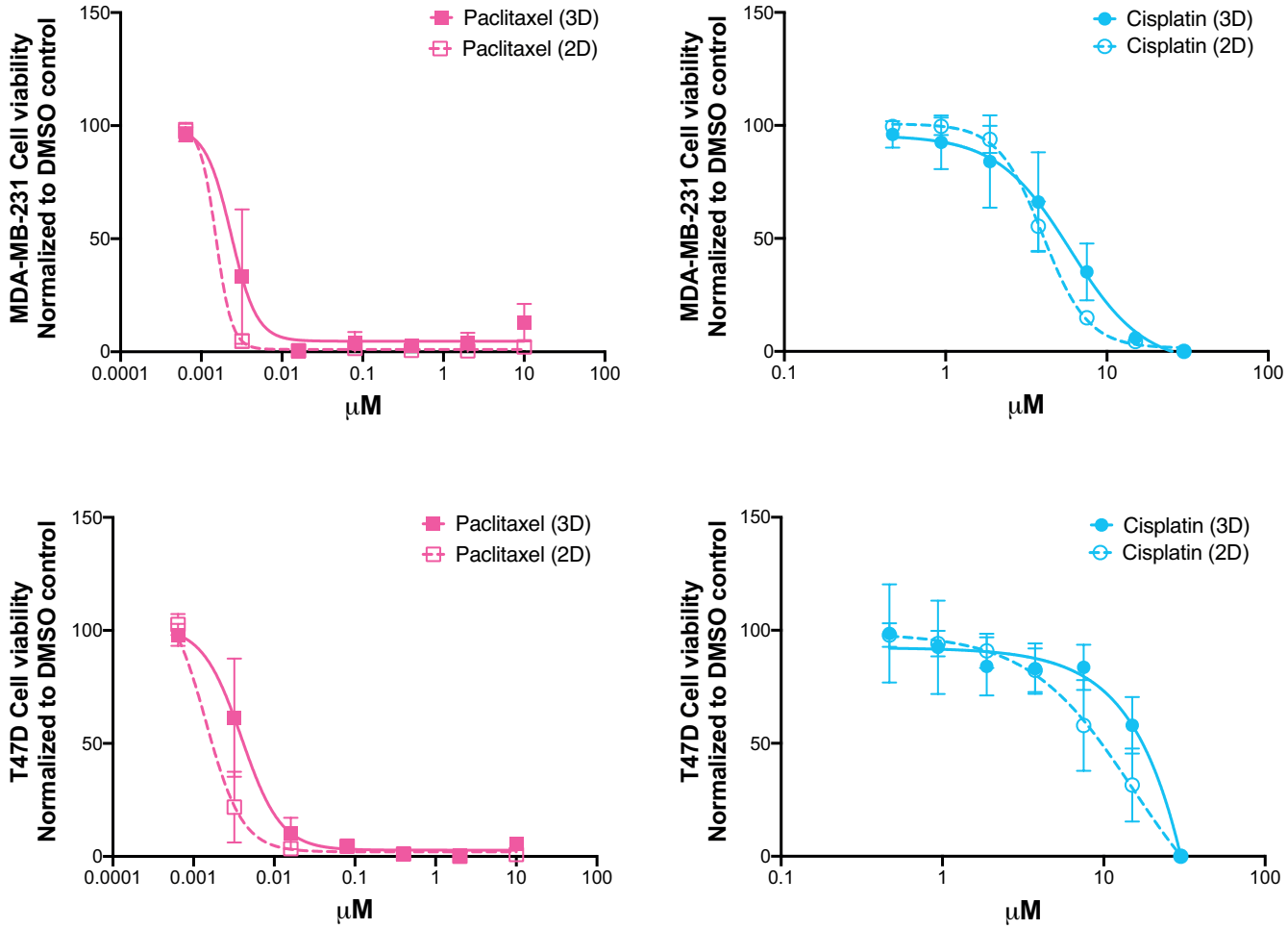


Figure 5: Response of breast cancer cell lines to paclitaxel and cisplatin. Human MDA-MB-231 and T47D cell lines were cultured in standard 2D conditions or using the r-Breast platform for 3 days, and subsequently treated with various concentrations of either paclitaxel or cisplatin for a further 7 days. Response to treatment was measured using the CellTiter Glo luminescence assay on a Spark Cyto.

CellTiter Glo 3D luminescence assay protocol (96-well plates):

1. Equilibrate the plate at room temperature for approximately 30 min.
2. Remove 140 µl of media from each well without disturbing the matrix, leaving ~60 µl of media in the well (assuming a total starting volume of 200 µl/well).
3. Add a 100 µl of CellTiter Glo 3D reagent per well, according to the manufacturer's protocol.
4. Mix the contents vigorously by repeated aspiration and dispensing to lyse the cells.
5. Incubate the plate at room temperature for a further 25 min to stabilize the signal.
6. Read the luminescence signal on the Spark Cyto with a 100 ms integration time, applying the OD=1 neutral density filter to attenuate the high intensity light.

LIVE/DEAD and CellTiter Glo can be combined into a multiplex assay that provides a comprehensive visual and quantitative representation of cellular behavior with and without therapeutic intervention. However, to obtain the data from both assays, the experimental set-up has to be doubled, increasing the costs and duration of each experiment. A method for sequential LIVE/DEAD and CellTiter Glo analysis of the same sample using the Spark Cyto was therefore established. For this, cells were initially stained as per the *in situ* LIVE/DEAD staining and imaging protocol, followed by CellTiter Glo analysis, as described previously.

Figure 6 shows the CellTiter Glo measurements after *in situ* LIVE/DEAD staining using either standard 2D culture conditions or the r-mBreast 3D culture platform. For 2D cultures, no significant difference was observed between the sequential (LIVE/DEAD followed by CellTiter Glo) or singleplex (CellTiter Glo only) assays. In contrast, cells grown in the 3D r-Breast matrix showed a 20-40 % reduction in luminescence signal when the multiplex methodology was used.

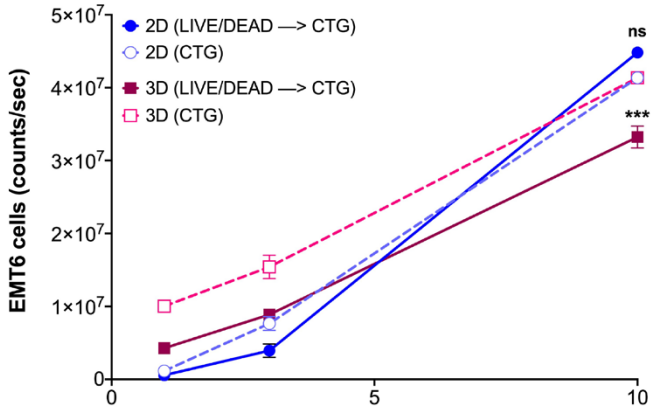


Figure 6: Comparison of sequential LIVE/DEAD/CellTiter Glo and CellTiter Glo analyses in mouse breast cancer cell lines grown in 3D or 2D systems. EMT6 cells were grown in standard 2D conditions or in r-mBreast cultures for 1, 3, and 10 days, then stained and analyzed according to the LIVE/DEAD protocol followed by the CellTiter Glo, or analyzed using the CellTiter Glo assay alone. There were no statistically significant differences in luminescence readouts between 2D cultures subjected to the multiplex protocol or CellTiter Glo alone. In the 3D system, a ≥20% reduction in signal intensity was observed when cells were sequentially subjected to LIVE/DEAD and CellTiter Glo protocols compared to CellTiter Glo alone.

CONCLUSIONS

The data presented here demonstrates that the Spark Cyto reader can be a useful tool for analyzing cells grown in 3D cultures using systems – such as r-Breast platform – where the culture architecture is dependent on the cell phenotype, with structures ranging from spheroids or stellate structures to cellular networks. In addition, the Spark Cyto can be used to test cell viability in response to treatment and, thanks to its temperature and carbon dioxide controls, real time imaging of 3D cultures.

Based on the analysis described above, the multiplexed, sequential protocol – where LIVE/DEAD staining was followed by CellTiter Glo analysis – offer time- and cost-savings for experimental set-ups where the luminescence signal of the 3D cultures is sufficient for analysis despite the observed reduction in intensity. Overall, the multimode capabilities of the Spark Cyto are particularly well suited to running multiplex protocols to test cellular responses in complex functional assays.

ABOUT THE AUTHOR

Julia Kirshner, Ph.D., Founder & CEO, zPREDICTA, Inc.

Julia has over 20 years of experience in oncology R&D spanning multiple areas of cancer biology, including: the development of organotypic preclinical models, molecular target and biomarker discovery, evaluation of drug-resistance, tumor microenvironment, cancer stem cells, and tumor immunology. Prior to founding zPREDICTA, Julia held a faculty appointment at the Department of Biological Sciences at Purdue University working on the mechanisms of cancer development and spread and the effects of tissue microenvironment on drug response. Subsequently, Julia founded zPREDICTA with a goal to develop tumor-specific 3D culture models reconstructing the native microenvironment of human tissues to mimic the drug behavior as seen in patients. From target discovery and lead optimization to preclinical evaluation of efficacy and toxicity, zPREDICTA's goal is to develop the tools necessary to accurately identify compounds that will have the highest probability of improving human health.

Spark Cyto is for research use only.

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