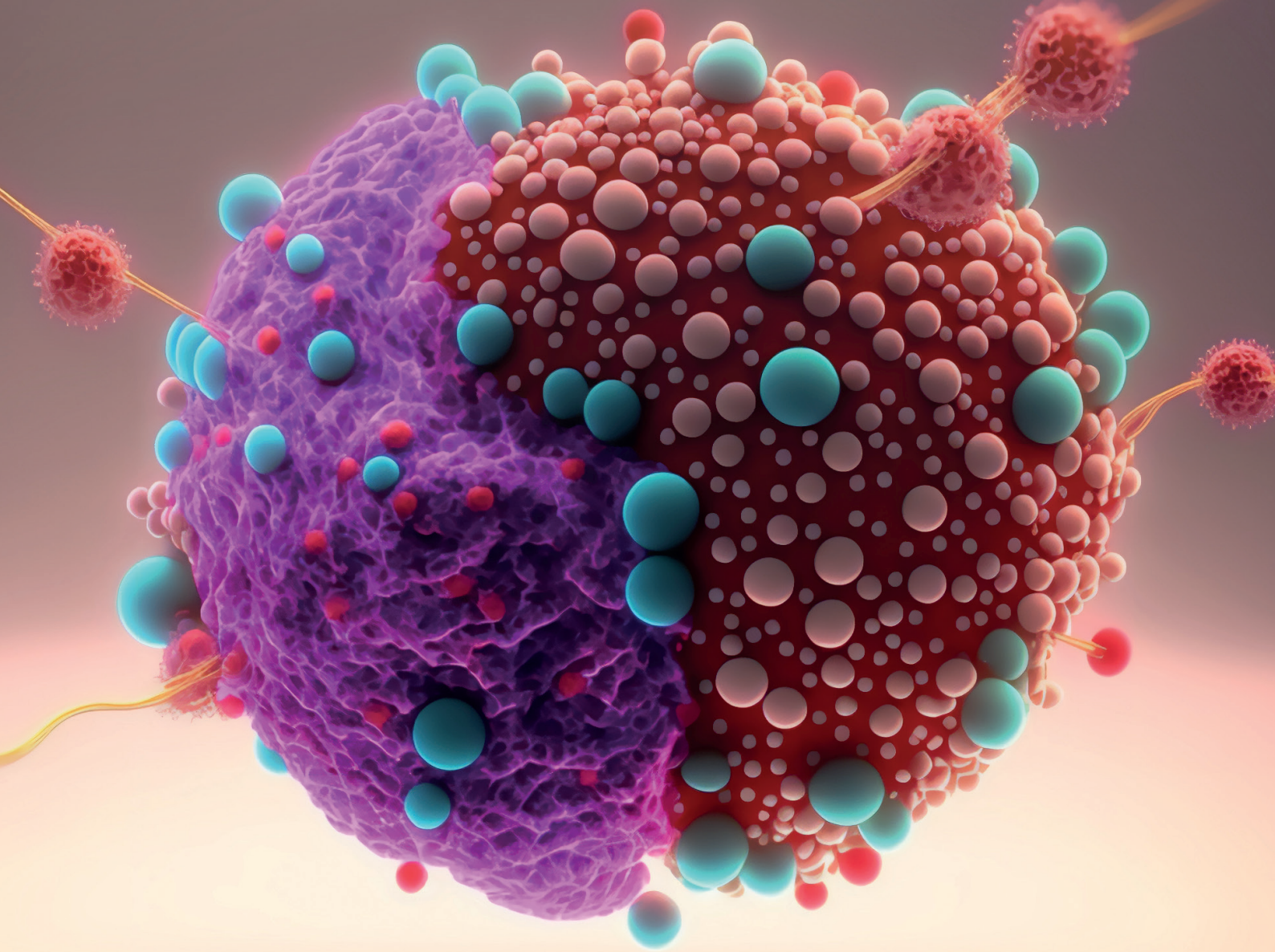
APPLICATION NOTE

IN VITRO CO-CULTURE AND IMMUNO-ONCOLOGY ANALYSIS OF HUMAN BREAST CANCER WITH THE R-BREAST 3D PLATFORM.

Using the Spark® Cyto for multiplex applications in 3D cancer models



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Introduction

Interactions between tumor cells, extracellular matrix (ECM), stromal cells, immune cells and secreted factors create a protective microenvironment that can impact the effectiveness of therapeutics. As a result, co-culture systems, which involve growing different cell types together in a controlled environment, have become an essential tool in breast cancer research. These systems allow scientists to simulate the interactions between cancer cells and other cells in the tumor microenvironment – such as immune cells and fibroblasts – which play critical roles in the development and progression of the disease. Studying breast cancer cells in co-culture with other cell types allows researchers to gain a better understanding of the molecular mechanisms driving disease, and identify potential therapeutic targets. Immuno-oncology, which focuses on harnessing the power of the immune system to fight cancer, is also a rapidly growing field in breast cancer research. Studying the interactions between immune cells and cancer cells in co-culture systems enables researchers to develop new immunotherapies targeting specific molecules or pathways involved in immune evasion by cancer cells.

Co-culture systems and immuno-oncology approaches ultimately have the potential to lead to more effective and personalized treatments for breast cancer patients. However, conventional methods of growing cells in flat layers on media surfaces may not accurately reflect how cancer cells respond to treatment, as these models lack the complex tumor microenvironment. The physiological tissue organization provided by 3D cell culture has the potential to deliver a more robust prediction of clinical outcome.

Predictive Oncology is a science-driven company offering solutions that introduce human diversity at an earlier stage of the drug discovery process. The company has developed a 3D culture model, r-Breast, to replicate the conditions in the mammary tissue and recreate the tumor microenvironment, including the epithelial and stromal niches. This provides a physiologically-relevant approach to evaluating new drugs or drug combinations, while also accounting for species-specific differences in the extracellular matrix composition.

3D techniques are more time consuming and expensive compared to 2D cell culture, so it is beneficial to perform multiplexed assays that increase the amount of information gathered within one experiment. This application note describes a study of two different co-culture systems using breast fibroblasts and T-cells. First, fibroblasts were co-cultured with breast cancer cells to assess the impact on drug sensitivity. Second, the effect of programmed cell death protein 1 (PD-1) drug treatment on the viability of breast cancer cells in the presence of T-cells was investigated. Cell viability was assessed using the r-Breast model with propidium iodide-based assays and CellTiter-Glo® luminescence analysis (Promega), efficiently evaluating the results using the Spark Cyto multimode reader.

MATERIALS AND METHODS

Materials

- Spark Cyto multimode reader (Tecan)
- CellTiter-Glo 2.0 Cell Viability Assay (Promega)
- Propidium iodide (Thermo Fisher Scientific)
- r-Breast 3D breast cancer culture model (Predictive Oncology)
- MDA-MB-231 human breast cancer cell line (ATCC)
- T-47D human breast cancer cell line (ATCC)
- Pembrolizumab (Thermo Fisher Scientific)
- Paclitaxel (Adipogen)
- Cisplatin (EMD Millipore)
- Human cancer-associated fibroblasts (BioIVT)
- Hoechst (Thermo Fisher Scientific)
- Propidium Idodide (Thermo Fisher Scientific)
- PBMC (ATCC)

r-Breast co-culture set-up

The tissue culture plates are initially coated with a specially designed matrix that mimics the stromal component of mammary tissue. The breast cancer cell lines are then combined with the r-Breast ECM and layered onto the stromal ECM on the plates. The mixture is allowed to solidify, forming a 3D layer measuring approximately 800 to 1,000 µm. Subsequently, growth medium enriched with specific factors relevant to tumor development is introduced to the cultures. For more intricate tumor environment simulations, stromal cells or cancer-associated fibroblasts (CAFs) can be included in the 3D layer to establish co-cultures between epithelial and stromal cells. After a period of 3 to 5 days, the breast cancer cells organize themselves into complex tissue structures, closely resembling their behavior in vivo. Some cell lines generate spheroids, while others form star-shaped or interconnected structures.

Once the 3D cultures have been established, various therapeutic agents – such as small molecules, antibodies, antibody-drug conjugates, bi-/tri-specific agents, and CAR-T cells – can be applied to the cells. The responses to these treatments can then be assessed directly within the cultures using techniques such as microscopy, CellTiter-Glo assays and immunohistochemistry. Additionally, the cells or spheroids can be separated from the r-Breast ECM through non-enzymatic digestion for downstream applications such as flow cytometry, next generation sequencing, or transplantation into animal models.

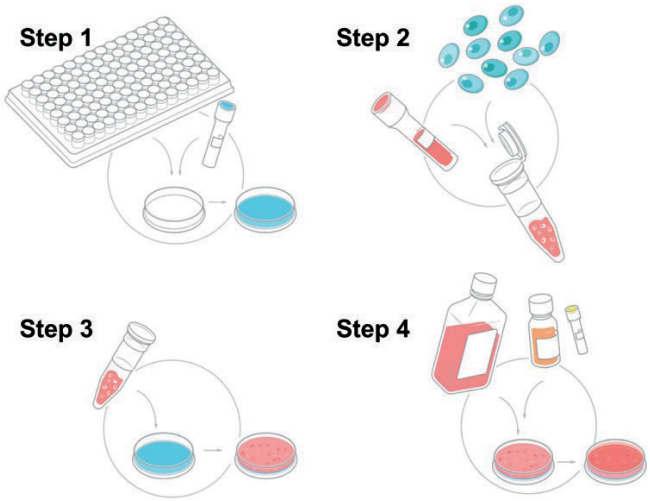

Figure 1: The r-Breast 3D co-culture set-up involves a simple, four-step process. 1) Coat plates with stromal ECM. 2) Mix cells with r-Breast ECM and overlay stromal ECM. 3) Polymerize. 4) Add growth medium with disease-specific supplements.

Propidium iodide positive cell imaging in r-Breast 3D cultures

A notable benefit of Predictive Oncology's 3D culture platforms is the ability to sustain cell cultures for extended durations. This allows researchers to examine the effects of drugs with different rates of action, including both fast- and slow-acting therapeutics. The propidium iodide (PI) viability assay is a reliable method for assessing cell survival in intricate culture structures over time, as well as for investigating changes in cell viability resulting from anticancer treatments.

In this study, the PD-1 inhibitor pembrolizumab was investigated for its effect on the viability of two breast cancer cell lines – MDA-MB-231 (triple negative) and T-47D estrogen receptor-positive/progesterone receptor-positive (ER+PR+) – co-cultured with T-cells. T-cells were pre-bound to pembrolizumab and co-cultured with breast cancer cell lines in 96-well plates. The breast cancer and T-cells were seeded in two different proportions (BCa:T 3:0 and BCa:T 3:1), and pembrolizumab was administered to the culture media at different concentrations. After 8 days of culturing, the cells were stained with Hoechst (461 nm emission) and PI dyes, and assessed for viability using the following protocol:

1. Remove culture media without disturbing the ECM matrix.
2. Add 100 µl/well of PI staining solution (10 µg/ml).
3. Incubate for 30 min at room temperature in the dark.
4. Aspirate the PI solution from each well and wash the cells twice with PBS to remove any unbound dye.
5. Add 100 µl of fresh medium to each well.
6. Image the cells in the Spark Cyto using the green (for GFP-labeled) and red optical filter sets within 1 hour of staining. For 3D cultures, set the focus offset to 300-400 µm, and exposure time to 10-50 ms, using the 10x objective.
7. ImageJ software was used to analyze the images acquired and quantify the proportion of PI-stained and unstained cells.

CellTiter-Glo analysis

The CellTiter-Glo assay is a well-established method to assess cell proliferation, establish growth curves and measure cytotoxic or antiproliferative effects of certain therapeutics. It was used in this study to demonstrate the potency of chemotherapy drugs. MDA-MB-231 and T-47D breast cancer cell lines were cultured with and without breast fibroblasts in the r-Breast 3D matrix for 3 days, and subsequently treated with various concentrations of either paclitaxel or cisplatin for a further 7 days.

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 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 25 min to stabilize the signal.
6. Read the luminescence signal on the Spark Cyto reader using an integration time of 100 ms and applying the OD=1 neutral density filter to attenuate the high intensity light.

RESULTS AND DISCUSSION

PI imaging and analysis

The assessment of T47D-GFP and MDA-MB-231-GFP cell viability in the presence of activated T-cells, mediated by the PD-1 inhibitor pembrolizumab, provides compelling evidence of T-cell induced cancer cell death. Viability staining employing PI reveals a PD-1 inhibitor dose-dependent increase in the proportion of dead cells, seen as an increase in the abundance of red dots in Figure 2. These observations demonstrate the efficacy of PD-1 blockade in promoting T-cell mediated elimination of cancer cells, highlighting its potential as a therapeutic strategy for cancer immunotherapy.

The proportion of PI-stained and unstained cells was determined using ImageJ analysis software, and this data was used to plot the effects of pembrolizumab on cell viability using GraphPad software (Figure 3), showing a twofold increase in the number of dead cells in the presence of the PD-1 drug.

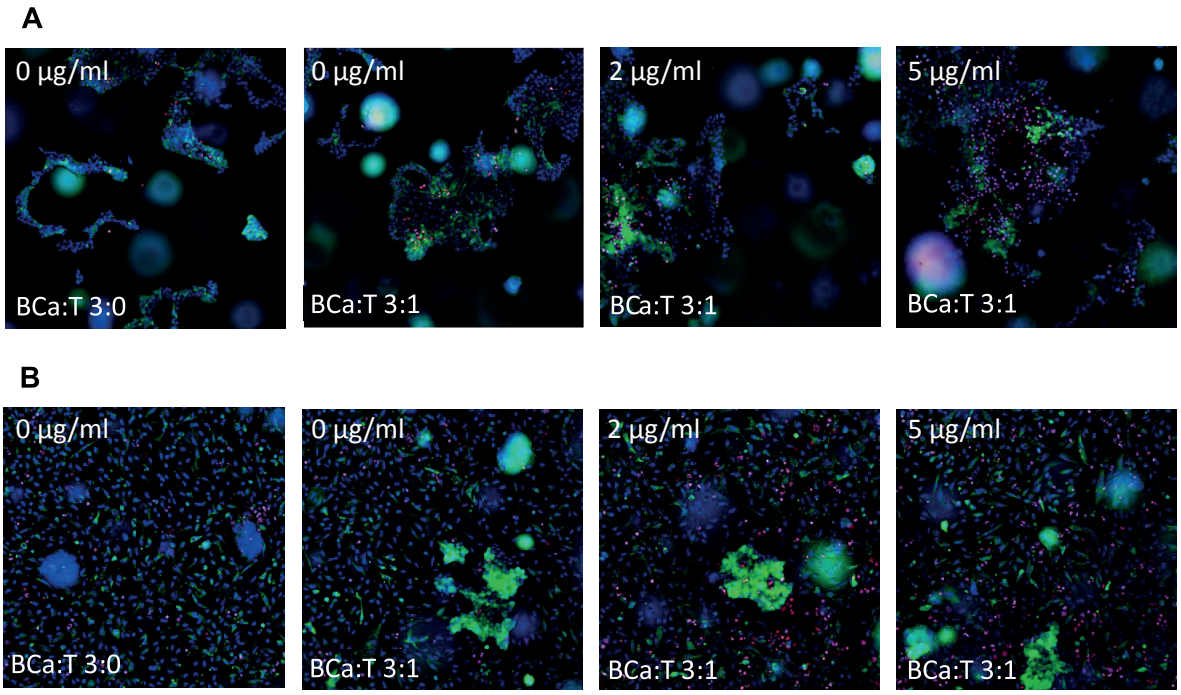

Figure 2: Viability staining of (A) T47D-GFP and (B) MDA-MB-231-GFP cell lines co-cultured with pre-activated T-cells.

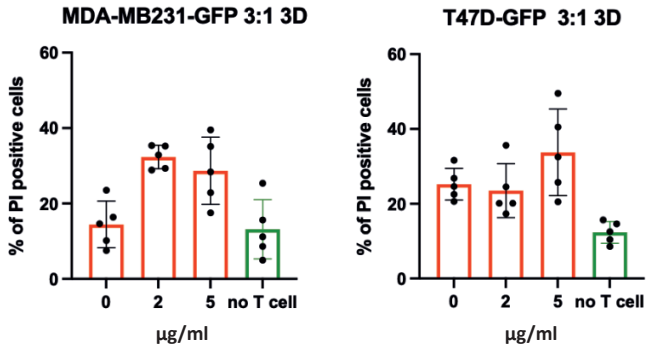

Figure 3: PI positive (dead cell) analysis of the breast cancer cell lines co-cultured with T-cells and treated with different concentrations of pembrolizumab.

CellTiter-Glo analysis

Both cell lines showed a higher sensitivity to paclitaxel than to cisplatin treatment (Figure 4). Cells cultured in the presence of breast fibroblasts using the r-Breast platform were more resistant to treatment with either compound compared to cells cultured without fibroblasts. This is presumably due to better mimicking of the tumor microenvironment in the r-Breast 3D model co-cultured with fibroblasts, which offers a degree of protection to tumor cells.

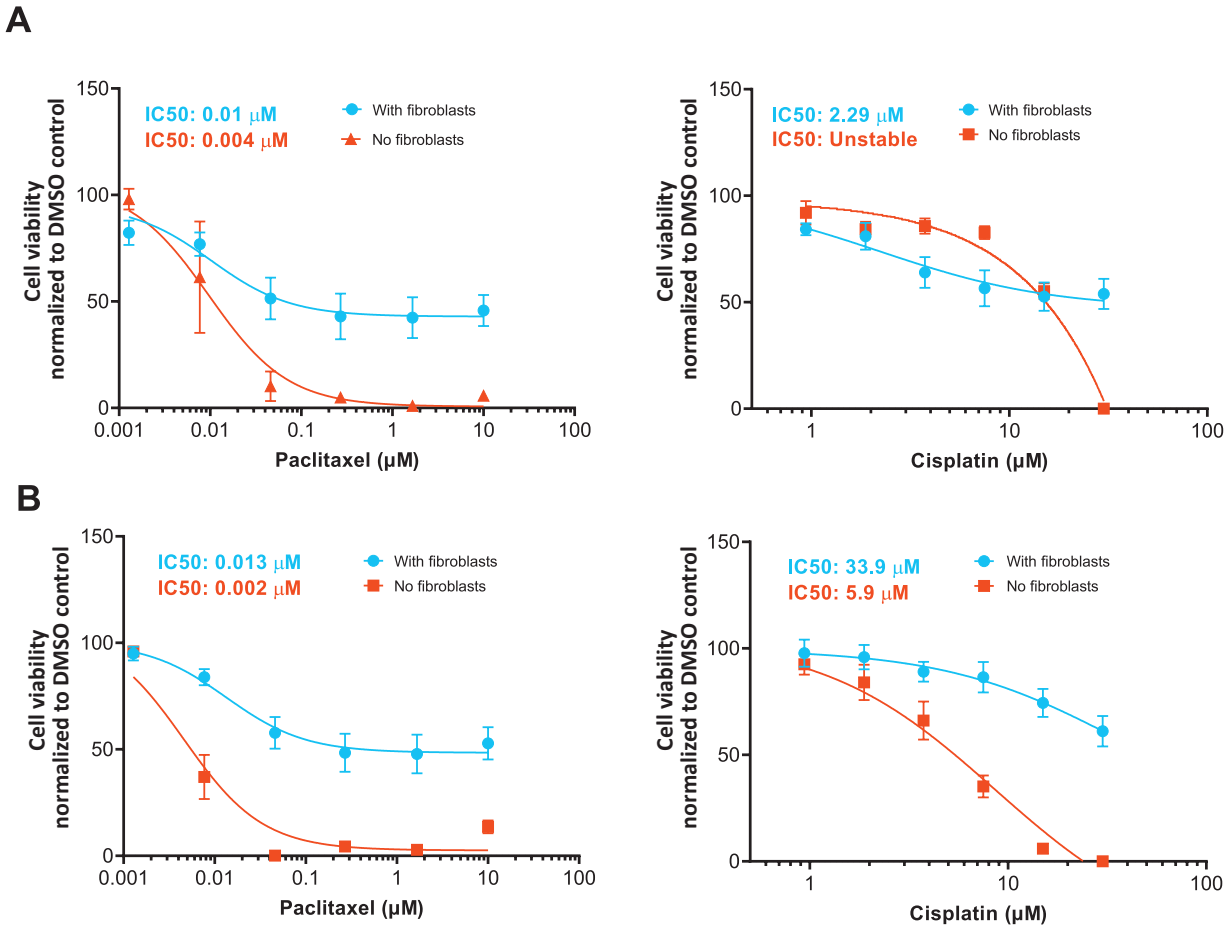

Figure 4: Response of (A) T-47D and (B) MDA-MB-231 human breast cancer cell lines co-cultured with or without breast fibroblasts to paclitaxel and cisplatin. Treatment response was measured using the CellTiter-Glo luminescence assay and a Spark Cyto reader.

Conclusion

The results presented in this study highlight the importance of using the r-Breast platform to investigate breast cancer cells in co-culture with other cell types exposed to chemotherapeutics. The culture architecture relies on the cell characteristics, resulting in diverse structures ranging from spheroids to stellate formations and cellular networks. The findings of this study will contribute to the understanding of the role of pembrolizumab in the treatment of breast cancer, and demonstrate that cells cultured in the presence of breast fibroblasts using the r-Breast platform were more resistant to treatment. These findings may have implications for the development of novel oncology drugs and immunotherapies.

The Spark Cyto reader enabled multiplexed analysis to examine cell viability by PI staining, followed by the CellTiter-Glo luminescence assay. The versatility of the Spark Cyto makes it ideally suited for conducting this type of multiplex protocol, reducing costs and saving time by enabling the examination of cellular responses in complex functional assays within one experiment.

About the authors

Arnat Balabiyev *joined Predictive Oncology in 2021 and is the lead scientist on 3D tumor models. He received his BS degree from Purdue University, and his PhD from Arizona State University. As part of his graduate studies, Arnat completed extensive research on cellular mechanism of macrophage fusion and its effect on foreign body reaction to biomaterials.*

Amy Ewing *joined Predictive Oncology in 2008 and is the Director of Scientific Program Delivery as well as a senior staff scientist. Amy holds a BS degree in biology from the University of Pittsburgh, and her various areas of expertise include cell culture, sequencing, assay development and validation, and regulatory affairs.*

Arlette Uihlein, MD, *joined Predictive Oncology in 2011 and serves as Senior Vice President for Translational Medicine and Drug Discovery, and Medical Director of the company's clinical and research labs. She received her medical degree from the Medical College of Ohio, and is board certified in anatomic and clinical pathology, and cytopathology. Her areas of expertise include cell culturing, high throughput drug screening, new assay development and validation, tissue imaging, molecular pathology, and applied Artificial Intelligence (AI).*

Magdalena Eckschlager *is an application specialist at Tecan Austria. She studied tissue engineering and regenerative medicine at FH Technikum in Vienna, and has worked in the field of embryonic development, with focus on stem cell differentiation in 3D model systems. Magdalena joined the sales and marketing team at Tecan in 2020, and has prioritized work related to the Spark Cyto multimode microplate reader.*

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402687 V1.0, 2023-09

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