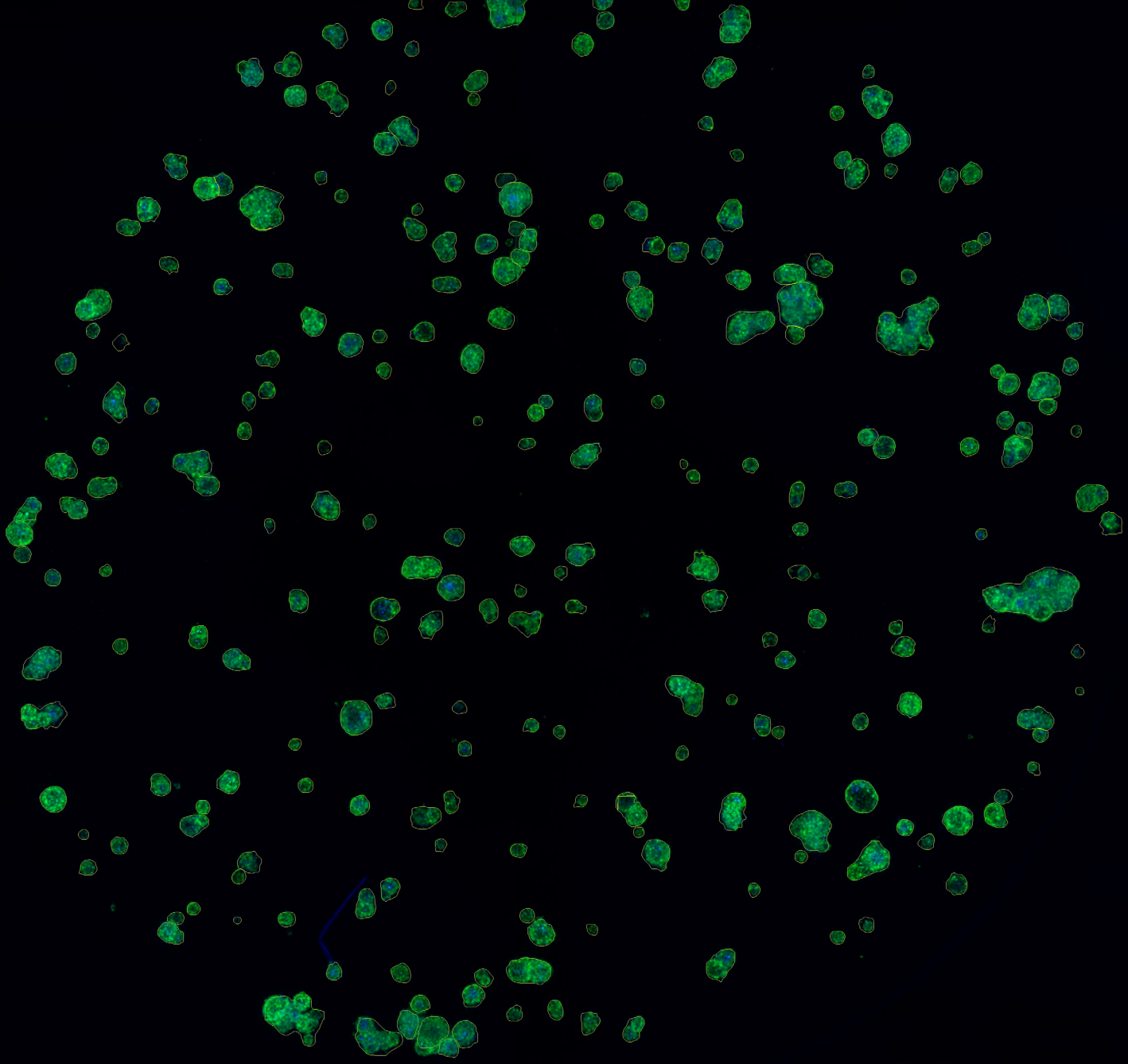
APPLICATION NOTE

ASSESSING DRUG RESPONSE IN PATIENT-DERIVED COLON ORGANOIDS.

Label-free, AI-based organoid analysis and drug response assessments using the Spark® Cyto multimode plate reader.



TECAN.

INTRODUCTION.

The development of drug treatments for human diseases is largely dependent on cancer-derived cell lines and animal models. These systems have inherent limitations, resulting in poor predictive values and clinical outcomes. Patient-derived organoids (PDOs) are in vitro models originating from normal or cancerous stem cells that preserve the original cellular complexity, tissue morphology and pathophysiology. Access to patient-matched normal and tumor PDOs enables the assessment of the tumor-specific activity and determination of the therapeutic window for compounds in development. This allows informed decisions early in the drug development pipeline, significantly improving the chances of success in clinical trials. Performing clinical studies using PDOs as a tool for personalized medical decisions – to predict patients' responses to therapeutic regimens – can significantly improve the treatment outcomes.

Currently, working with PDOs can be challenging due to the lack of automated equipment suitable for performing high throughput, imaging-based drug screening in organoid analysis pipelines. Tecan is addressing this need with a deep learning-based algorithm for the Spark® Cyto multimode plate reader, allowing automatic identification and segmentation of objects with complex 3D structures, such as organoids or spheroids.

This application note evaluates the Spark Cyto for automated PDO imaging and analysis, using the new 3D^{ai} deep learning-based algorithm available in the SparkControl™ software, as well as optimization of settings and re-analysis of the images in the Image Analyzer™ software. PDOs cultured and compound-treated in 96- and 384-well Corning® Ultra-Low Attachment (ULA) plates were imaged using the brightfield and green and blue fluorescence channels. CyQUANT™ dye was used in combination with a nuclear stain to visualize the samples and evaluate the cytotoxicity of the drug-treated PDOs. SparkControl and Image Analyzer were used to perform real-time image analysis and downstream verification and optimization of results.

MATERIALS AND METHODS.

Organoid culture.

A colorectal cancer (CRC) PDO model, derived from adult stems cells, showing a compact morphology, was used in this study. The PDOs were passaged every 7 days by mechanical shearing, and cultured for at least two passages, or until there were enough PDOs to initiate the experiment.

List of additional materials.

Material	Provider
Staurosporine, 10 mM	Merck, 37095
Fluorouracil, 5 mg/ml	Accord, 15596885
Cultrex® Basement Membrane Extract extracellular matrix	Bio-Techne, 1678650
Colon Tumor Medium	HUB
Dispase II neutral protease, 100 mg/ml (100x)	Thermo Fisher, 17105041
CyQUANT™ Cell Proliferation Assay	Thermo Fisher, C7026
Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent, 1x	Life Technologies, R37605
R-spondin 3 Wnt agonist, 2.26 mg/ml	Bio-Techne, TAQ382203A
Wnt3aCM medium supplement, 50 ml	N.A.
Y-27632 ROCK inhibitor, 10 mM	AbMole, M1817
384-well plates, black/clear bottom, ULA	Corning, 4588
96-well plates, clear, ULA	Corning, 3474
pluriStrainer® 40 µm cell strainer	pluriSelect, 43-50040-51
pluriStrainer 100 µm cell strainer	pluriSelect, 43-50100-51
Spark Cyto 600	Tecan
Fluent® Automation Workstation	Tecan

PDO viability assay.

PDO viability was assessed using the CyQUANT Cell Proliferation Assay to measure organoid viability. PDOs were split one day before the start of the assay. On the day of the experiment, Basement Membrane Extract (BME) domes were digested by adding 100 mg/ml of dispase II to the wells, followed by incubation for at least 30 minutes at 37 °C, 5 % CO_2. The PDOs were then filtered through a 100 µm pluriStrainer. The strainer was washed with Ad-DF+++ (Advanced DMEM/F12 medium supplemented with 2 mM glutamax, 10 mM HEPES and 100 µg/ml penicillin-streptomycin) containing

10 µM Rho-kinase inhibitor, and the flowthrough was filtered through a 40 µm pluriStrainer. The PDOs retained on the 40 µm strainer (size ranging from 40 µm to 100 µm) were collected in organoid medium supplemented with 10 µM ROCK inhibitor. NucBlue 1:20 was added to the organoid suspension, which was then incubated for 30 minutes at 37 °C, 5 % CO_2. The PDOs were subsequently washed with Ad-DF+++, counted, and plated at a density of 250 organoids in 40 µl of 5 % BME suspension in 384-well plates, or 500 organoids in 100 µl of 5 % BME suspension in 96-well plates.

PDOs were treated with either 5-fluorouracil (5-FU) – the current standard-of-care compound – or staurosporine (STS) as positive control, then added to the 384-well and 96-well plates using a Fluent Automation Workstation. Plates were incubated at 37 °C, 5 % CO_2 for 5 days. Images of the organoids were acquired on days 0, 2 and 5 after seeding using the Spark Cyto. Z-stack 0-400 µm with 40 µm step width was used in combination with the instrument's 4x objective and the brightfield channel. On day 5, CyQUANT reagent was added to the PDOs using the Fluent workstation. Fluorescent and brightfield images were collected 4-6 hours after CyQUANT addition using the Spark Cyto, and image analysis was performed using SparkControl and Image Analyzer software. Notably, the dose-response curves were generated using mean CyQUANT intensity and % viability based on brightfield image segmentation, showing the strength of Tecan's new deep learning-based algorithm in terms of label-free organoid segmentation and analysis.

RESULTS AND DISCUSSION.

Figures 1 and 2 show representative images of CRC PDOs cultured in 384-well and 96-well plates, respectively.

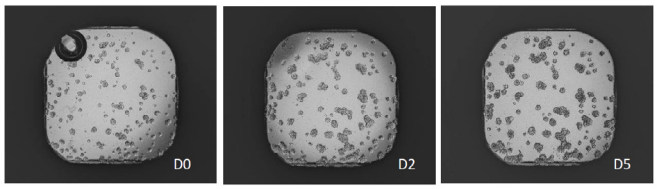


Figure 1: Representative brightfield images of untreated CRC PDOs cultured in a 384-well plate at day 0 (D0), day 2 (D2) and day 5 (D5) post-seeding. Images were acquired with the Spark Cyto using the 4x objective and a whole well field of view.

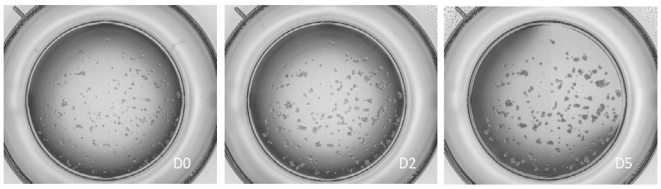


Figure 2: Representative brightfield images of untreated CRC PDOs cultured in a 96-well plate at day 0 (D0), day 2 (D2) and day 5 (D5) post-seeding. Images were acquired with the Spark Cyto using the 4x objective and a whole well field of view.

PDOs were then exposed to 10 point concentration titration of 5-FU, and the drug response was assessed using the Spark Cyto's automated organoid imaging and analysis pipeline. Figures 3 and 4 show the corresponding dose response curves in the 384-well and 96-well formats, respectively.

$$PDO\ \%viability = \frac{Cyquant\ intensity\ sample - Cyquant\ intensity\ STS\ control}{Cyquant\ intensity\ PBS\ control - Cyquant\ intensity\ STS\ control} * 100\%$$

Equation 1: Equation to calculate PDO viability in %.

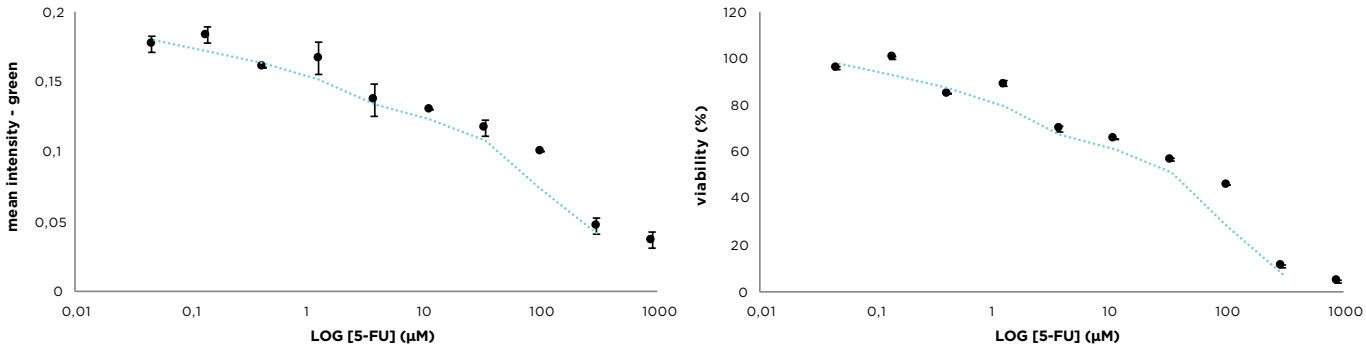


Figure 3: 5-FU dose response curves of PDOs 5 days after seeding in a 384-well plate. Left: mean CyQUANT intensity. Right: % viability.

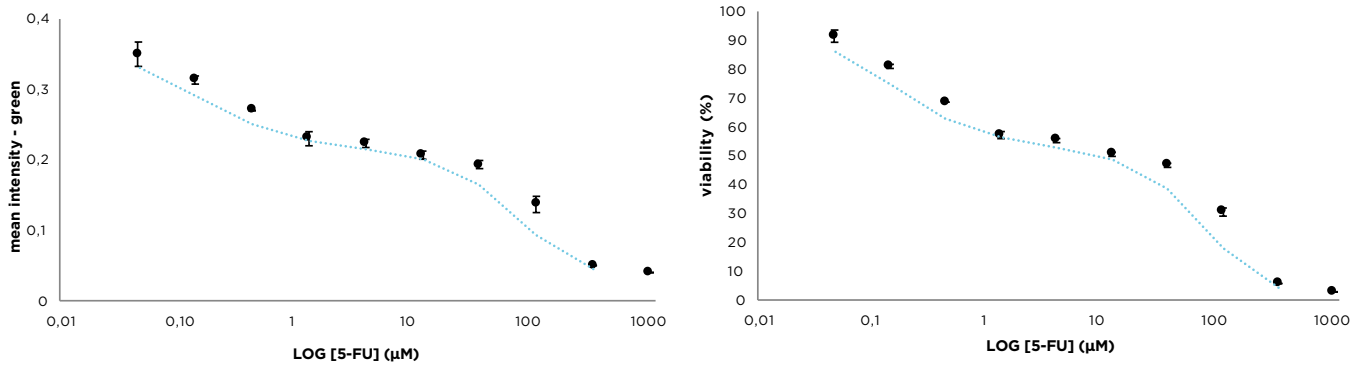


Figure 4: 5-FU dose response curves of PDOs 5 days after seeding in a 96-well plate. Left: mean CyQUANT mean intensity. Right: % viability.

A representative image (Figure 5) shows the accurate segmentation of PDOs in the 384-well plate, with only some organoids on the outer edges of the plate not recognized by the deep learning-based algorithm. This can be the case if some objects are outside of the applied z-range. Further optimization of the z-range used for image acquisition might help to include all objects during analysis, but requires confirmation in future experiments.

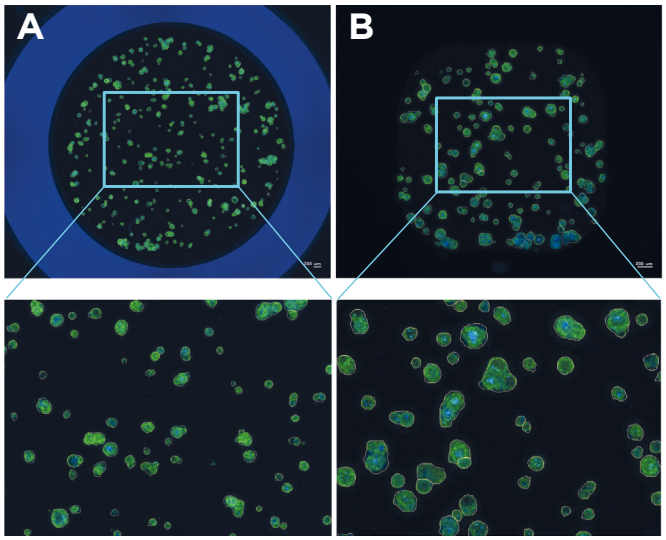


Figure 5: Representative fluorescent images with segmentation (zoom) of untreated CRC PDOs in (A) a 96-well or (B) a 384-well plate 5 days after seeding. The image was acquired with the Spark Cyto using the 4x objective.

CONCLUSION.

In this study, CRC PDOs cultured in 96- and 384-well ULA plates were successfully imaged and analyzed in all experimental conditions. The Spark Cyto's imaging and analysis pipeline using SparkControl and Image Analyzer software successfully identified almost all objects. In certain instances, partially fused PODs may be segmented as distinct objects by the algorithm. Enhancing the identification of objects near the edges of the wells can be achieved by optimizing image acquisition parameters for z-stacking, including adjustments to the z-range and step width. However, drug response tests generated results in line with expectations in both 96-well and 384-well plate formats, demonstrating the Spark Cyto instrument's ability to perform automated, high throughput organoid imaging and analysis.

SparkControl software allows users to easily set up 3D imaging methods with a user-friendly interface. It provides real-time image analysis, offering full control and feasibility during the instrument's operation. Results of image analysis can be effortlessly visualized with the intuitive Image Analyzer software, enabling one-click setting optimization for re-analysis, such as the 'object gating' function or z-stack alterations. Finally, the software allows users to export the results to Microsoft Excel®, and provides a detailed PDF report. This easy-to-use and user-friendly approach automates drug response assessments in organoids, increasing the overall efficiency of drug development and personalized medicine initiatives.

ACKNOWLEDGMENTS.

We express our acknowledgments to HUB Organoids. HUB Organoids is the global leader in organoid technology. Their mission is to develop the world leading patient-derived organoid based platform to enhance the efficiency and effectiveness of pharmaceutical research by closely replicating human organ physiology.

Their advanced organoid models enable pharmaceutical and biotech companies to conduct high-throughput screening, toxicology studies, and disease modeling with unparalleled patient-relevance. By mimicking the complexities of human tissues, HUB Organoids empowers researchers to better predict drug responses, streamline preclinical testing, and minimize reliance on traditional animal models.

ABBREVIATIONS.

BME	Basement membrane extract
CRC	Colorectal cancer
CTM	Colon tumor medium
DMEM	Dulbecco's Modified Eagle's Medium
FGF10	Fibroblast growth factor 10
NA	Not applicable
5-FU	5-Fluorouracil
HEPES	4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid
PBS	Phosphate-buffered saline
PDO	Patient-derived organoids
PGE2	Prostaglandin E2
ROCK inhibitor	Rho-kinase inhibitor
STS	Staurosporine
ULA	Ultra-low attachment
Wnt/ Wnt3a	Wingless-related integration site

ABOUT THE AUTHORS.

Yasmine Abouleila, PhD *is a Senior Scientist and Project Leader at HUB Organoids. She completed her PhD in Biomedical and Health Sciences at Hiroshima University, Japan, where she focused on identifying new diagnostic biomarkers for early cancer detection through circulating tumor cells. Since joining the oncology department at HUB Organoids in 2022, she has been responsible for leading and designing several cancer research and immuno-oncology projects. She oversees all stages of these projects, with a primary focus on testing and validating new drug targets and treatments.*

Eric Lutsch, MSc. *is an Application Scientist at Tecan Austria. He studied medical biology at the University of Salzburg, where he achieved his master's degree. His work focused on regenerative biology and aging, conducting stem cell research, and establishing workflows for 3D cell culture, such as spheroids. Eric has experience in 3D cell culture, tissue engineering, microfluidics, and low cost device design and production. He joined the sales and marketing team at Tecan in 2023, with an emphasis on the Spark Cyto multimode reader and its imaging and detection applications.*

Christian Oberdanner*, PhD is a Senior Marketing Application Scientist at Tecan Austria. He studied cell and molecular biology to PhD level at the University of Salzburg, with a strong focus on tumor biology. Christian started working for Tecan Austria as an external scientific consultant in 2005, and joined the company permanently in 2006. Since then, he has held several roles, including Application Scientist, Application Specialist, and Product Manager. Christian's priorities within Tecan are multimode microplate reader applications and cell imaging.*

For Research Use Only. Not for use in diagnostic procedures.

Australia +61 3 9647 4100 **Austria** +43 62 46 89 330 **Belgium** +32 15 42 13 19 **China** +86 21 220 63 206 **France** +33 4 72 76 04 80 **Germany** +49 79 51 94 170
Italy +39 02 92 44 790 **Japan** +81 44 556 73 11 **Netherlands** +31 18 34 48 17 4 **Nordic** +46 8 750 39 40 **Singapore** +65 644 41 886 **South Korea** +82 2 818 3301
Spain +34 93 595 25 31 **Switzerland** +41 44 922 89 22 **UK** +44 118 9300 300 **USA** +1 408 782 5600 **Other countries** +41 44 922 81 11

403054 V1.0, 2024-10

www.tecan.com