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Label-Free, Real-Time Live Cell Assays for 3D Organoids Embedded in Matrigel®

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Introduction

Advances in preclinical *in-vitro* models are crucial for both basic research and drug development across a range of applications. Organoid technologies are increasingly being used as *in-vitro* models of human development and disease as they exhibit structural, morphogenetic and functional properties that recapitulate *in-vivo* pathophysiology.[1] To successfully use these models across a variety of research disciplines and applications, approaches that reduce variability and technology pipelines to image and quantify these complex cell models are required.

Currently, techniques to robustly characterize and visualize these models may be limited by one or more of the following:

- Time-consuming, expensive or laborious acquisition processes.
- Use of third party analysis software.
- Random, end point assessments or indirect (e.g. ATP) readouts that may overlook key morphological changes over time.
- Requirement to label cells (fluorescence-based quantification), which may be challenging and not amenable to a range of cell types.

The Incucyte® Organoid Analysis Software Module provides a solution to standardize and automate organoid acquisition and analysis workflows, simplifying characterization of these complex cultures.

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Assay Principle

This application note describes the use of the Incucyte® Live-Cell Analysis System along with the Incucyte® Organoid Analysis Software Module to study the growth or death of organoids, label-free. A proprietary brightfield image acquisition approach enables real-time kinetic imaging of 3D organoids embedded within a matrix (Matrigel®). Organoid size, count and morphology measurements are automatically plotted over time to gain in-depth organoid characterization following perturbation. Here we describe validation methods and data demonstrating the ability to kinetically image and quantify the growth, death and morphology of organoids embedded in Matrigel®.

Materials and Methods

Quick Guide

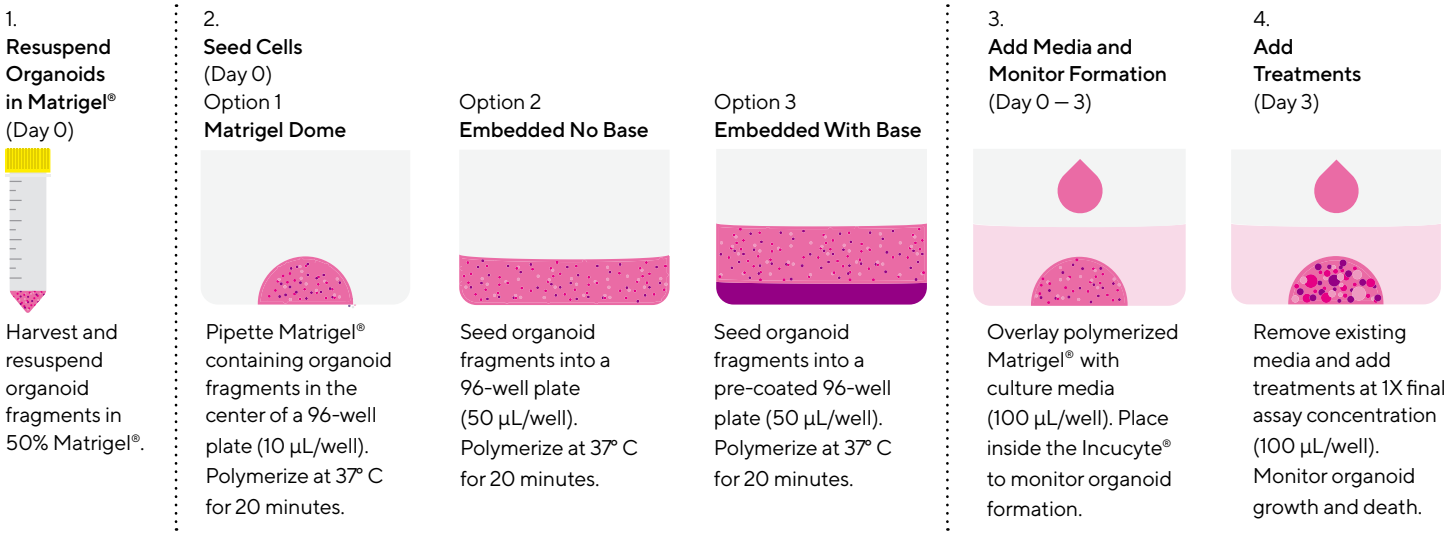

Figure 1: Incucyte® Organoid Assay Workflow

1. Organoids of interest are harvested according to model-specific guidelines and resuspended in 50% Matrigel®.
2. Matrigel® containing organoid fragments is pipetted into each well of a 96-well tissue culture treated plate utilizing any of following assay formats:
   - **Matrigel® Dome** (Pre-warmed plate; 10 µL/well)
   - **Embedded No Base** (Pre-chilled plate; 50 µL/well)
   - **Embedded With Base** (Pre-coated plate; 50 µL/well)
3. Plate is placed in a humidified incubator to polymerize Matrigel® (37° C, 20 minutes).
4. Cell type-specific growth media is added on top of polymerized Matrigel® (100 µL/well).
5. Organoid formation is monitored in an Incucyte® (Organoid Assay scan type, 4x, 6-hour repeat scanning, 0 – 3 d).
6. Post formation, treatments are added (100 µL at 1x final assay concentration (FAC) per well).
7. Organoid growth and death is monitored within an Incucyte® every 6 hours for up to 10 days. Organoid metrics (e.g. size, count, eccentricity) are reported in real-time based on brightfield image analysis.

Organoid culture reagents were obtained from StemCell Technologies unless otherwise noted. Mouse intestinal (#70931), hepatic (#70932), human brain (healthy or patient derived; prepared externally) and human lung organoids (cultured by University of California San Diego[2]) were embedded in Matrigel® (Corning #356231 or #354277 brain organoids) in 96-well flat bottom TC-treated microplates (Corning #3595).

Organoids were cultured in cell type-specific organoid growth medium (e.g. IntestiCult™ OGM Cat. #06005; #06040; HepatiCult™ OGM #06030; STEMdiff™ Cerebral Organoid Kit Cat. #08570) supplemented with 100 units/100 µg per mL Pen/Strep (Life technologies). Organoid formation, growth and death was monitored in an Incucyte® at 6-hour intervals for up to 10 days.

Visualizing and Quantifying Differential Organoid Phenotypes in a 96-Well Assay Format

To evaluate the ability of the Incucyte® Organoid Analysis Software Module to accurately track organoid growth in 96-well plates, mouse intestinal, hepatic, or human whole lung organoids[2] were embedded in Matrigel® (50 %) and bright-field (BF) images were acquired every 6 hours (Figure 2A).

Organoids were automatically located and changes in size (area) were kinetically tracked using Incucyte' s Organoid Software Analysis Module. BF area segmentation shown in yellow (Figure 2) enabled label-free quantification of organoid growth and illustrates the software's ability to accurately segment individual objects embedded in Matrigel® across a range of cell types.

Acquired BF images (6 d post seeding) and time-courses (Figure 2A) revealed cell type-specific morphological features and temporal growth profiles respectively. Individual lung organoids appeared larger (400 µm – 1 mm diameter) than Intestinal (100 - 400 µm) or hepatic (50 - 500 µm) organoids and reached maximal size rapidly (90 h, 22.1 x 10^4 µm² ± 2.5 mean ± SEM, n = 3 wells).

To further demonstrate the software's ability to distinguish organoid morphological differences, human brain organoids derived from healthy- or epilepsy- iPSCs (induced pluripotent stem cells) were embedded in 50% Matrigel® and imaged over 8 days (Figure 2B). Figure 2B illustrates that these cultures exhibited comparable growth (avg. area bar chart) but displayed distinct phenotypes (BF images). Morphology-related parameters tracking changes in object roundness (eccentricity) or brightness (darkness) were utilized to exemplify differential organoid phenotypes. Mature healthy organoids appeared darker and rounded (decreased eccentricity), while an increase in eccentricity was observed in patient organoids as they formed loose, disorganized aggregates (Figure 2B).

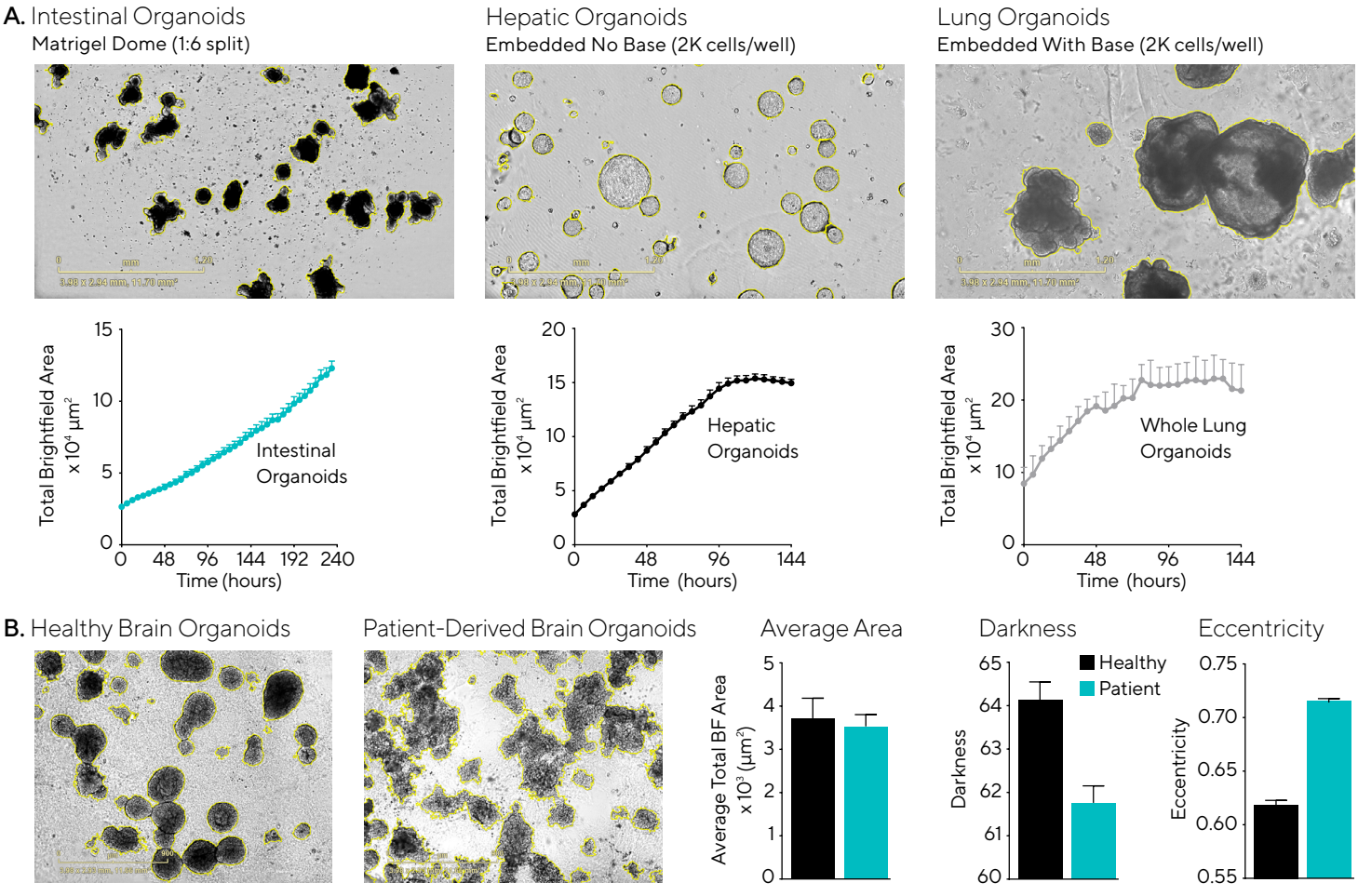

Figure 2: Acquisition and quantification of distinct organoid morphologies. Mouse intestinal (1:6 split, dome), hepatic (2K cells/well) and whole lung organoids (2K cells/well) were seeded (50% Matrigel®) into 96-well plates and imaged in an Incucyte®. Brightfield (BF) images (6 days post seeding) and time-course plots of the individual well total BF area (µm²) over time (hours) show distinct organoid phenotypes and demonstrate cell type specific organoid growth, respectively (A). Healthy or diseased human brain organoids (2K cells/well) were embedded in 50% Matrigel® and imaged over 8 days. Images (6 days) and bar graphs demonstrate growth capabilities and differential phenotypes of healthy vs diseased organoids (B). All images captured at 4x magnification. Each data point represents mean ± SEM, n = 3 - 12 wells.

Quantifying Organoid Growth and Death Over Time

To assess the impact of treatments on organoid growth and morphology, intestinal and hepatic organoid fragments were embedded in Matrigel® (50%) and allowed to form organoids for 3 days prior to treatment with protein kinase inhibitor staurosporine (1 µM, STP). Changes in organoid size and shape were kinetically monitored and quantified over time (4 - 10 days).

Figure 3A demonstrates that vehicle treated intestinal or hepatic organoids increase in size (10-fold or 3-fold respectively) and number (Figure 3B) over time while a marked reduction is observed in the presence of STP.

Time-courses and zoomed in BF images shown in figure 3B illustrate the effect of STP on hepatic organoid morphology. A concomitant increase in darkness and eccentricity was observed as STP induced cell death and elicited loss of distinctive rounded phenotype over time.

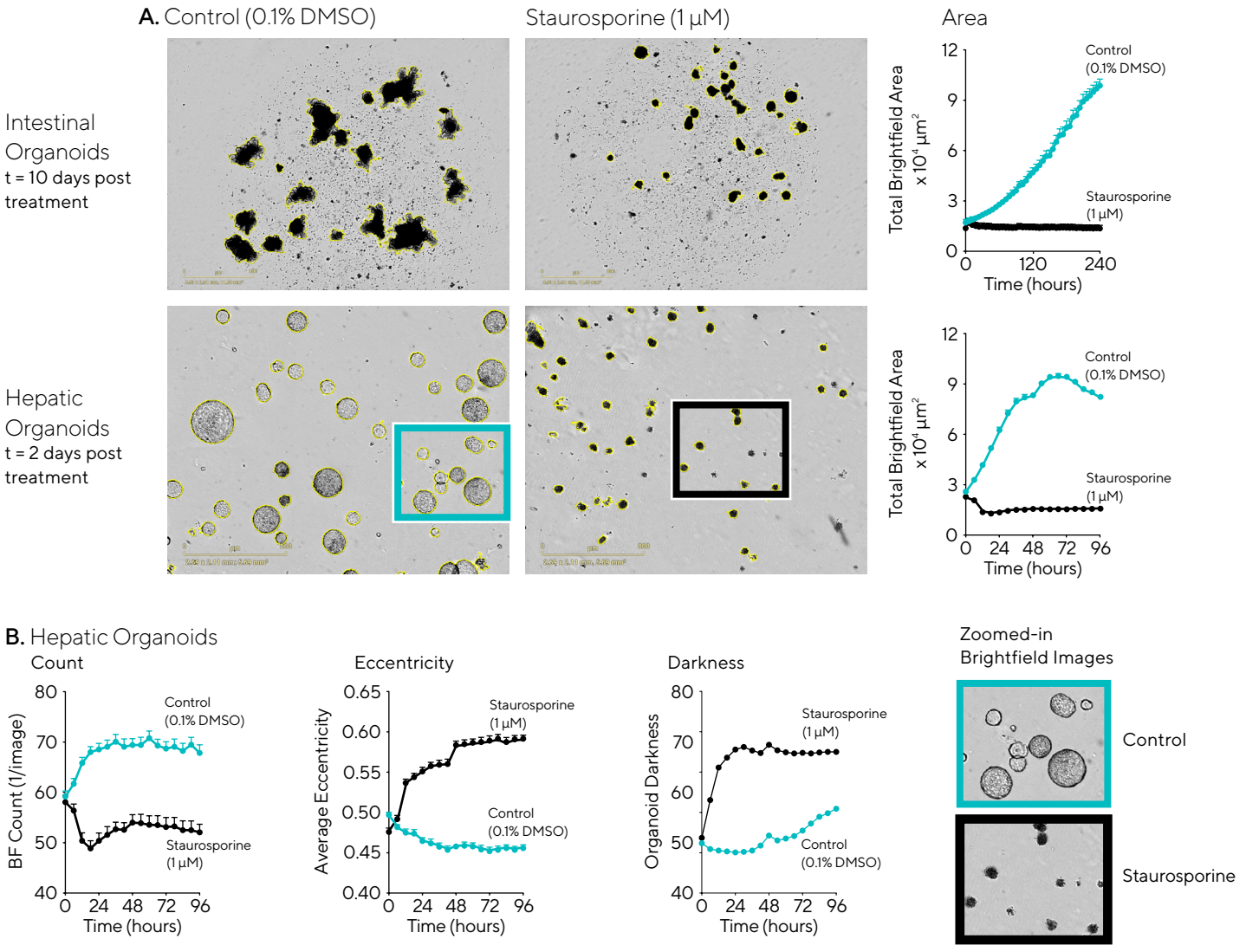

Figure 3: Perform automated label free quantification of organoids. Mouse intestinal (1:6 split) and hepatic fragments (1K cells/well) were embedded in Matrigel® (50%) in 96-well plates and allowed to form organoids for 3 days prior to treatment (vehicle or staurosporine; STP). Brightfield (BF) images (A) and corresponding time-courses of BF area (A) demonstrate the continued growth of vehicle treated organoids and the inhibitory effects of STP across both cell types. STP treated hepatic organoids lose distinctive rounded phenotype (increased eccentricity) and increase in darkness over time (B). Data were collected over a 96 - 240 hour period at 6 hour intervals. All images captured at 4x magnification. Each data point represents mean ± SEM, n=4 wells.

Probe Mechanisms of Action Using Real-Time Morphology Measurements

As patient-derived organoids (PDOs) retain the morphological and molecular characteristics of the tissue/tumour of origin, they are increasingly being used as *in-vitro* drug development models.[1] For these *in-vitro* drug studies, the ability to distinguish between cytotoxic and cytostatic cellular responses is crucial to establishing effective anticancer therapies.[3] Performing multi-parametric quantitative measurements is key to understanding these dynamic drug responses.

To exemplify drug-specific changes in organoids, hepatic organoids were formed for 3 days and subsequently treated with staurosporine (STP, protein kinase inhibitor), cisplatin (CIS, DNA synthesis inhibitor) or fluorouracil (5-FU, thymidylate synthetase inhibitor). Concentration response curves (CRCs) representing the area under the curve analysis of total area, eccentricity, or darkness time-course data (0 – 96 hours) were then constructed to discriminate between cytotoxic and cytostatic agents (Figure 4).

All compounds caused a concentration dependent inhibition of organoid growth, yielding IC_{50} values of 3 nM for STP, 0.78 µM for 5-FU and 9.7 µM for CIS (area CRC, Figure 4).

However, while attenuation of organoid size was observed across all compounds, increases in eccentricity and darkness indictive of 3D structure disruption and cell death respectively were only observed in CIS and STP-treated organoids.

STP induced notable changes in organoid eccentricity across a range of concentrations (1.6 nM – 1 µM, EC_{50} 0.5 nM) and evoked a concentration-dependent increase in organoid darkness (EC_{50} 53.3 nM), suggesting a strong cytotoxic mechanism of action (MoA). While concentration-dependent responses were also observed in CIS-treated organoids, substantially higher concentrations (50 -100 µM) were required to elicit comparable or greater effects on eccentricity (EC_{50} 32.5 µM) or darkness (EC_{50} 31.7 µM).

Conversely, 5-FU appeared to be more cytostatic, inhibiting organoid growth but not inducing cell death or disrupting distinct organoid phenotype. Differences between the size and morphology readouts support the cytostatic mechanism of 5-FU. Representative BF images confirm distinction between the cytotoxic MoA of STP and CIS and the cytostatic effects of 5-FU on hepatic organoids (Figure 4).

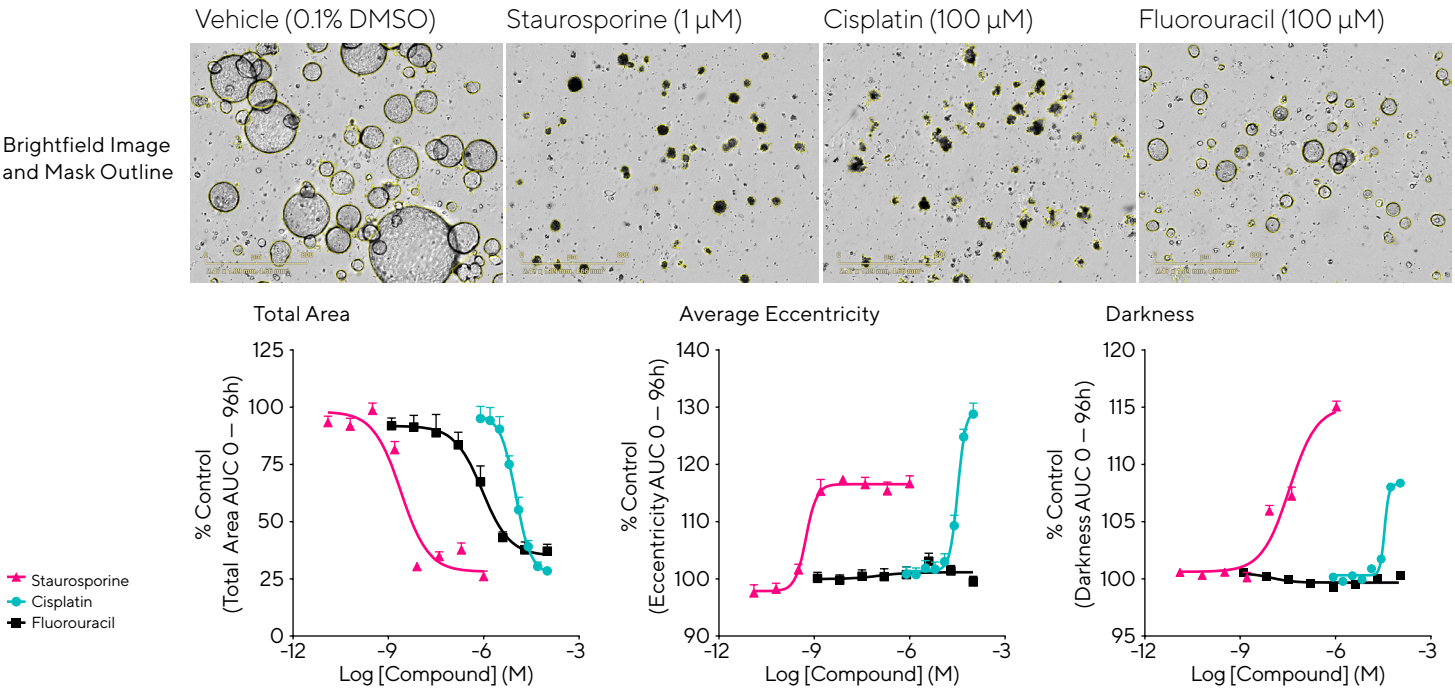

Figure 4: Distinguish between cytotoxic and cytostatic mechanisms of action using label-free measurements. Hepatic fragments were embedded (0.5K cells/well) in 50% Matrigel® and allowed to form organoids (3 days) prior to treatment. Brightfield images taken 2 day post treatment show compound-specific effects on organoid size and morphology. Concentration response curves (CRCs) of the area under the curve (AUC) analysis of area, eccentricity and darkness demonstrated differential profiles of cytotoxic (staurosporine and cisplatin) and cytostatic (fluorouracil) mechanisms of action. Data were collected over a 96-hour period at 6 hour intervals. Each data point represents mean ± SEM, n = 3 separate test occasions.

Label-free Quantification of Forskolin-Induced Organoid Swelling

Cystic fibrosis (CF) is caused by mutations in the CFTR gene that severely reduce the function of the anion channel, cystic fibrosis transmembrane conductance regulator (CFTR). As disease expression is highly variable between patients (>1,900 CFTR mutations), effective responses to treatment therapies are challenging.[4,5] Despite the promise of CFTR-specific drug therapies, the degree of individual CFTR function restoration has been limited by *in-vitro* screening models. However, numerous studies have recently highlighted the translational potential of organoids and demonstrated their use to successfully obtain patient-specific information on CFTR-modulator drug response.[5]

Here, we validate a rapid and label-free quantitative swelling assay for CFTR function in mouse intestinal organoids. Organoids formed for 3 days were treated with increasing concentrations of forskolin (Fsk; cyclic adenosine monophosphate inducing stimuli) and imaged in an Incucyte® every 15 — 20 minutes for up to 6 hours. Forskolin-induced swelling (FIS) was kinetically quantified by evaluating the percentage change in BF area (size) relative to the area at t = 0 hours.

Exposing intestinal organoids to forskolin caused a concentration-dependent increase in organoid size, while DMSO-treated organoids remained unchanged (Figure 5A and 5B). Note the 3-fold increase in size (6 hour post treatment) at the highest test concentration in comparison to control (Figure 5B). Additionally, as the lumen filled with fluid over time, stimulated organoids became more rounded and clearer, resulting in a reduction in eccentricity and darkness respectively (Figure 5C).

To demonstrate that FIS is CFTR-dependent and thereby mimicking the CF disease state, intestinal organoids were pre-incubated with CFTR inhibitor $CFTR_{inh}$-172 for 2 hours. The construction of concentration response curves revealed that swelling is CFTR-dependent as control organoids increased in size (>300% at 10 µM Fsk), while treatment with $CFTR_{inh}$-172 reduced the FIS maximal response by >50% (~150% at 10 µM Fsk, Figure 5D).

Validation data shown in figure 5 demonstrate the capability to kinetically visualise and quantify CFTR function label-free and illustrates the potential utility of this approach in cystic fibrosis drug development, diagnosis, or functional studies.

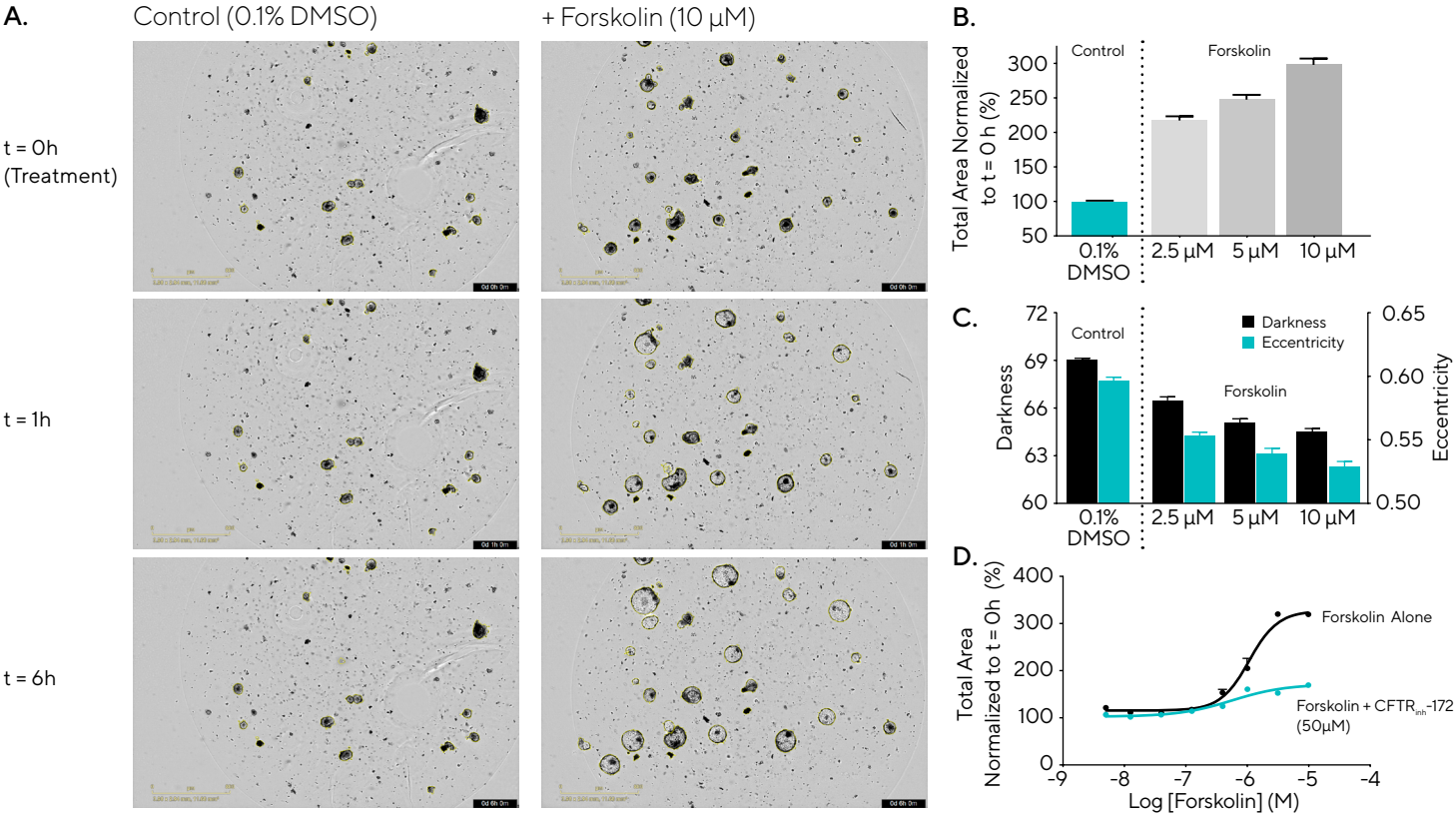

Figure 5: Quantify organoid swelling in response to Forskolin stimulation. Incucyte® brightfield (BF) images show effects of forskolin treatment on intestinal organoid (50% Matrigel® domes) size over time (A). Bar chart of BF area (total BF area normalized to t = 0 h) demonstrates that swelling is forskolin concentration-dependent (B). Following stimulation intestinal organoids exhibit a more rounded phenotype (decreased eccentricity) and clear lumen (decreased darkness) (C). Concentration response curve (CRC) of the area under the curve (AUC) analysis of area normalized to t = 0 hours (%) (0 – 6 hours) demonstrates that forskolin-induced swelling is cystic fibrosis transmembrane conductance regulator (CFTR)-dependent (D). Data were collected over a 6-hour period at 30 minute intervals. BF images captured at 4x magnification. Each data point represents mean ± SEM, n=3.

Conclusion

In this application note, we demonstrate the use of the Incucyte® Live-Cell Analysis System, in combination with the Incucyte® Organoid Analysis Software Module, to simplify and facilitate temporal assessment of organoid growth or death. We have demonstrated:

- Automated software that can continuously locate and analyze embedded organoids in physiologically relevant conditions.
- The ability to kinetically visualize and quantify distinct organoid morphologies and segment individual objects embedded in Matrigel® across a range of cell types.
- The use of integrated, real-time label-free metrics to assess drug-induced cellular changes and characterize mechanisms of action in a 96-well plate format.
- Use of this approach to visualize and quantify CFTR function, thereby enabling label-free assessment of cystic fibrosis *in-vitro*.

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