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Application Note

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Validation and Pharmacological Utility of Real-Time, Live-Cell Assays for Single 3D Spheroids

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Introduction

A growing body of evidence suggests that more relevant and translational observations can be made using 3D micro-tissues and organoids as compared to 2D monolayer cell models. This is most notable in the fields of cancer biology, immuno-oncology and hepatotoxicity.[1] 2D models, though cost effective, fail to replicate the 3D complexity of an *in vivo* tumor and associated influences of the tumor microenvironment, such as that of cell-to-cell contact and the influence of the extracellular matrix (ECM).[2] For example, 3D tumor spheroids exhibit more relevant morphology and increased cell survival as compared to 2D cultures. Spheroid models have a layered structure consisting of an external layer of more rapidly proliferating cells, and a quiescent zone and hypoxic necrotic core resulting from a gradient of nutrients, metabolites and oxygen, which are important attributes for evaluation of drug resistance due to penetration effects in a heterogeneous tumor.[2,3] Single spheroids formed in ultra-low attachment (ULA) plates are best suited to researchers wishing to study larger solid tumors, which may have hypoxic cores, and a requirement for high levels of well-to-well consistency. This approach also avoids the use of complex and poorly characterized biomatrices.

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Current methods for assessing the growth and shrinkage of 3D tumor spheroids are limited, however, by one or more of the following:

1. Assay workflows that are time-consuming, expensive or laborious
2. A requirement to label the cells (e.g., a fluorescent probe) that may perturb the biology and may not be amenable to primary tissue
3. Single time point readouts that do not report the full time course
4. Interruption of environmental control when cells are removed from the incubator for imaging
5. Indirect readouts (e.g., ATP) that may overlook valuable morphological insight and/or misreport cell growth

Assay Principle

In this application note, we describe methods and validation for miniaturized (96 | 384-well) Incucyte® Single Spheroid Assays based on non-invasive bright-field image analysis, performed with the Incucyte® Spheroid Analysis Software Module. The Incucyte® proprietary image acquisition technique, depth of focus (DF) Brightfield, was used to generate high contrast, extended depth of focus images in conjunction with the Incucyte® Single Spheroid Assay protocol. Consistent segmentation and analysis were performed with an automated image processing algorithm for data generation, which masked the largest brightfield (BF) object in the field and reduced operator bias. This enabled assessment of spheroid viability without the use of perturbing reagents. These assays were flexible, simple to run, employed non-perturbing and validated reagents, and provided automated imaging to monitor morphological changes and direct measurement of tumor size and health in real time. Tumor spheroids, formed in ULA plates, were placed within the incubator to maintain physiological conditions and monitored for up to two weeks to record kinetic changes.

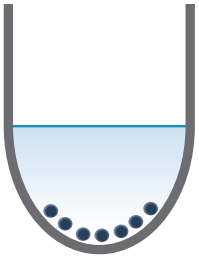
As described here, fluorescence characterization was achieved by determining fluorescence within a BF mask boundary, removing the requirement to set fluorescence masking parameters, such as threshold setting. The software analysis tools enabled unbiased analysis and robust data suitable for pharmacological analysis. Pharmacological utility was also explored further, and included demonstrated spheroid growth and shrinkage assays with robust intra- and inter-plate reproducibility. To demonstrate proof-of-concept for exploring mechanisms of drug action, cellular responses to both cytotoxic (camptothecin) and cytostatic (cycloheximide) agents were assessed in cells stably expressing Incucyte® Cytolight | Nuclight Lentivirus or Incucyte® Cell Health Reagents (Incucyte® Caspase 3/7 or Annexin V).

Taken together, the combination of the Incucyte® Single Spheroid Assays, Incucyte® reagents, and the Incucyte® Spheroid Analysis Software Module provide a more complete solution to overcome the current limitations of 3D spheroid models, allowing real-time kinetic assessment of the growth and shrinkage of 3D tumor spheroids with greater physiological relevance. This enables greater model understanding for the identification of drug targets, as well as the enhancement of pharmacological analysis and interpretation.

Materials and Methods

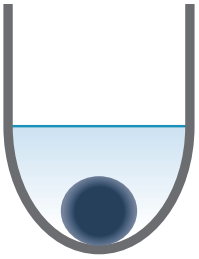
Quick Guide

1. Seed cells (day 0)



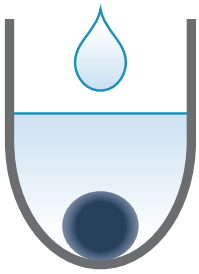
Seed cells into 96W or 384W ultra low attachment plate. Centrifuge.

2. Spheroid formation (day 0–3)



Place plate inside the Incucyte® and scan every six hours.

3. Add treatments (day 3)



Add treatments to plate. Monitor spheroid growth and shrinkage.

Figure 1: Assay workflow

1. Cells of interest are harvested, counted, and plated into ULA round-bottom 96- or 384-well plates at desired densities (in 100 or 50 µL per well for 96- or 384-well plate respectively). Plates are centrifuged (150 xg, 10 min). Seeding density will need to be optimized for each cell line used; recommended range of 1,000–5,000 cells per well (10,000–50,000 cells per mL seeding stock).
2. Spheroid formation is monitored to desired size (e.g., 200–500 µm in diameter) with Brightfield and HD phase-contrast image acquisition (either 4X or 10X magnification) every 6 h using Incucyte® Live-Cell Analysis System.
3. Optional: Incucyte® Cell Health Reagents are added either at formation or up to 3 days post seeding.
4. Addition of test compounds (100 µL at 2X, or 25 µL at 3X final assay concentration (FAC) per well for 96- or 384-well plate respectively).
5. The spheroid growth and shrinkage assay is initiated and monitored in Incucyte® Live-Cell Analysis System (6 h repeatedly scanned, up to 2 weeks). Tumor size is reported in real time based on Brightfield image analysis.

All cell culture reagents were obtained from Life Technologies unless otherwise noted. SKOV3 (EACC) cultures were stably transfected with Incucyte® Nuclight Red Lentivirus, EF1 Alpha Promoter, Puromycin selection, (Cat. No. 4625), prepared per Essen BioScience protocol. Incucyte® Nuclight Red Cellular Reagents (MDA-MB-231-NR,No. 4487; A549-NR Cat. No. 4491; HT-1080-NR, Cat. No. 4485) and SKOV3-NR cultures were grown to confluence in 75 cm^2 tissue culture treated flasks and seeded into 96-well (Corning, Cat. No. 7007) or 384-well ULA round-bottom plates (S-Bio , PrimeSurface® 3D Spheroid Plates, Cat. No. MS-9384UZ). Spheroids formed the desired size by 72 h, and the plates were centrifuged (150 xg) at room temperature for 10 min. MDA-MB-231 cells required the addition of Matrigel® Basement Membrane Matrix (Corning, Cat. No. 356234) at 2.5% v/v, to promote tight spheroid formation. All cells were cultured in F-12K supplemented with 10% FBS, 1% Pen | Strep, 1% glutamax and 0.5 µg/mL puromycin. Spheroid formation was monitored in an Incucyte® Live-Cell Analysis System over a 72 h period at 6 h intervals.

Where required, Incucyte® Cell Health Reagents were included to determine cytotoxicity (Incucyte® Cytotox Green or Red Dye, EssenBio Cat. No. 4633 or Cat. No. 4632), Caspase 3/7 activity (Incucyte® Caspase 3/7 Green or Red Dye, EssenBio Cat. No. 4440 or Cat. No. 4704) or phosphatydilserine externalization (Incucyte® Annexin V Green or Red Dye, EssenBio Cat. No. 4642 or 4641).

Validation Data

Spheroid Morphology

To illustrate the value of the Incucyte® Live-Cell Imaging and Analysis, human lung (A549) and breast tumor cell lines (MtDA-MB-231 in Matrigel®) were seeded at 5,000 or 2,500 cells per well, respectively, in 96-well ULA plates (Corning). High definition (HD)-phase and Brightfield (BF) images were taken at 6 h intervals (Figure 2). We observed clear morphological differences: A549 cells formed larger loose aggregates, while MDA-MB-231 spheroids were small and compact. These properties are consistent with those previously described elsewhere.

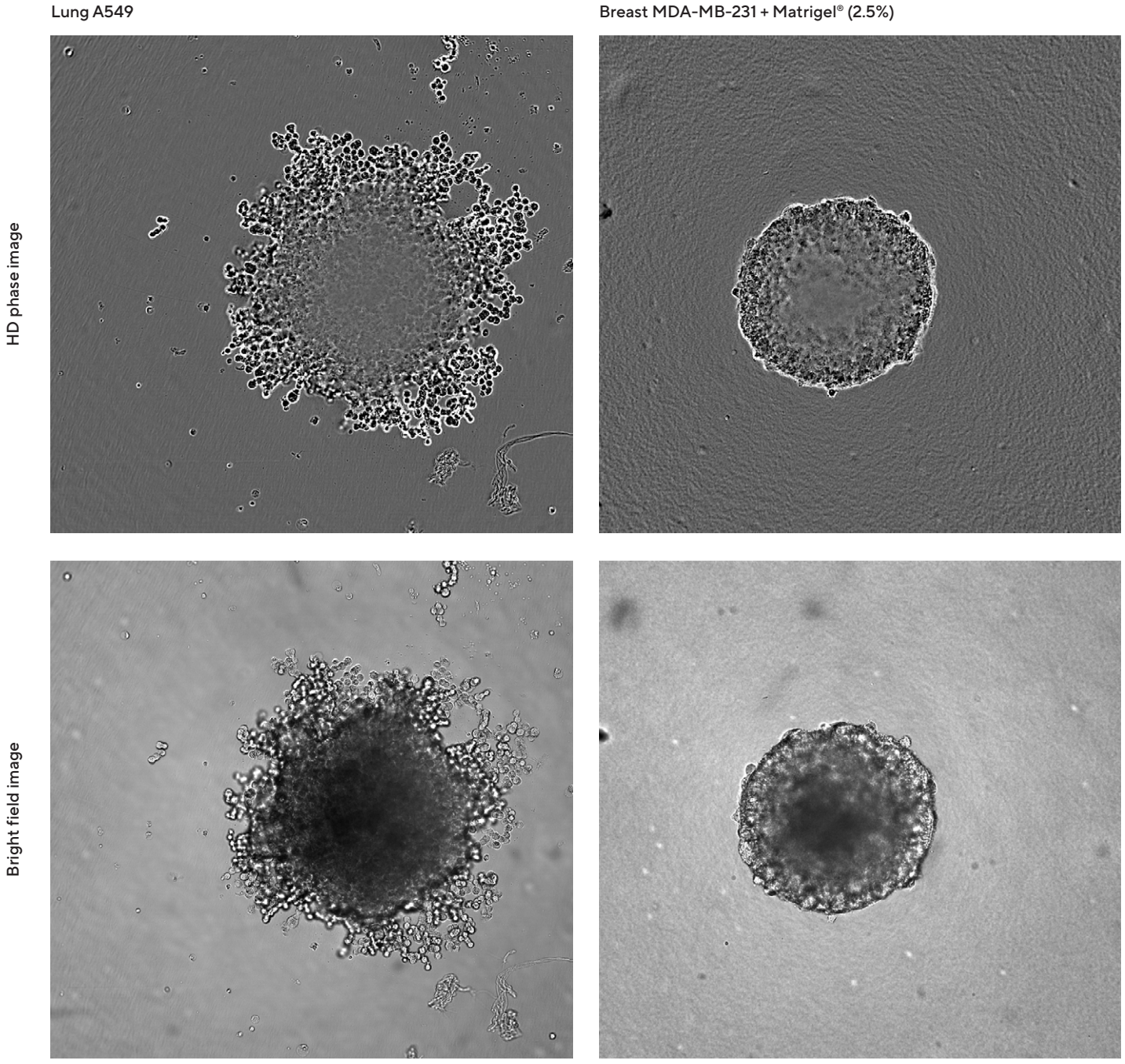


Figure 2: Visualizing different spheroid morphologies. High quality HD phase and corresponding BF images of spheroids formed from A549 and MDA-MB-231 cells (2,500 or 5000 cells per well, respectively), 72 h post seeding. Visualization of detailed phenotypic variation is observed in HD phase images. A549 cells present a loose aggregate morphology compared to the compact spheroid formed by MDA-MB-231 cells. Compaction of MDA-MB-231 aggregates into spheroids was achieved by the addition of 2.5% v/v Matrigel® post centrifugation. All images captured at 10X magnification.

Label-Free Kinetic Monitoring of Spheroid Growth

For validation of kinetic measurement capabilities, the size of tumor spheroids was measured over time using the Incucyte® DF Brightfield analysis automated software algorithm that masked the largest Brightfield object in the field of view. Changes in the size and morphology of MDA-MB-231, HT-1080 and A549 tumor spheroids were monitored over time for the creation of growth curves, in the absence and presence of the cytotoxic drug, camptothecin (CMP, Figure 3). For all three cell types, the control spheroids increased markedly over the 10-day period (2–8 fold)—the largest size increase was observed with MDA-MB-231 in Matrigel®. CMP inhibited spheroid growth, and in the case of HT-1080 cells, an overall reduction in spheroid size compared to the t = zero control was observed.

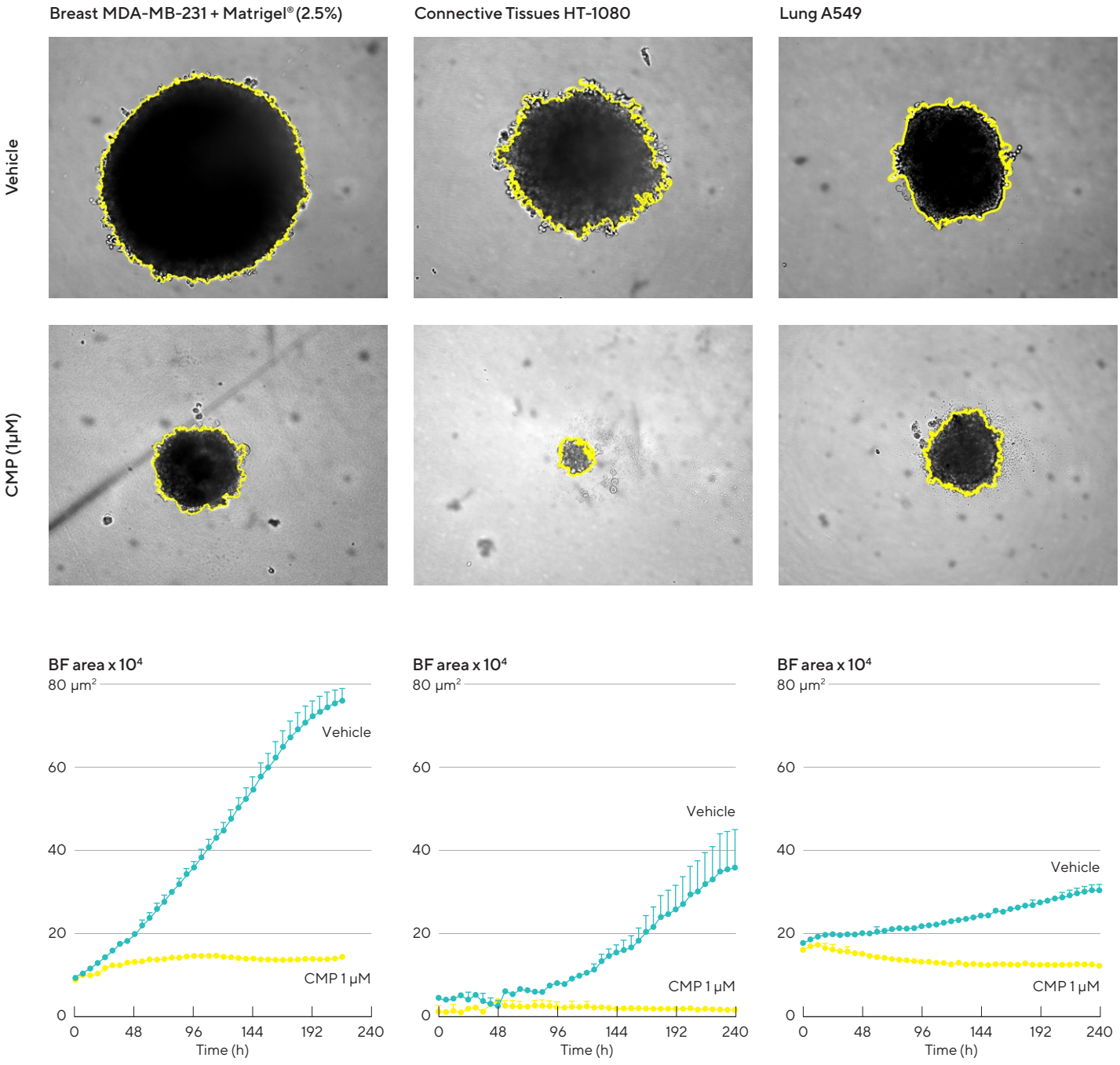
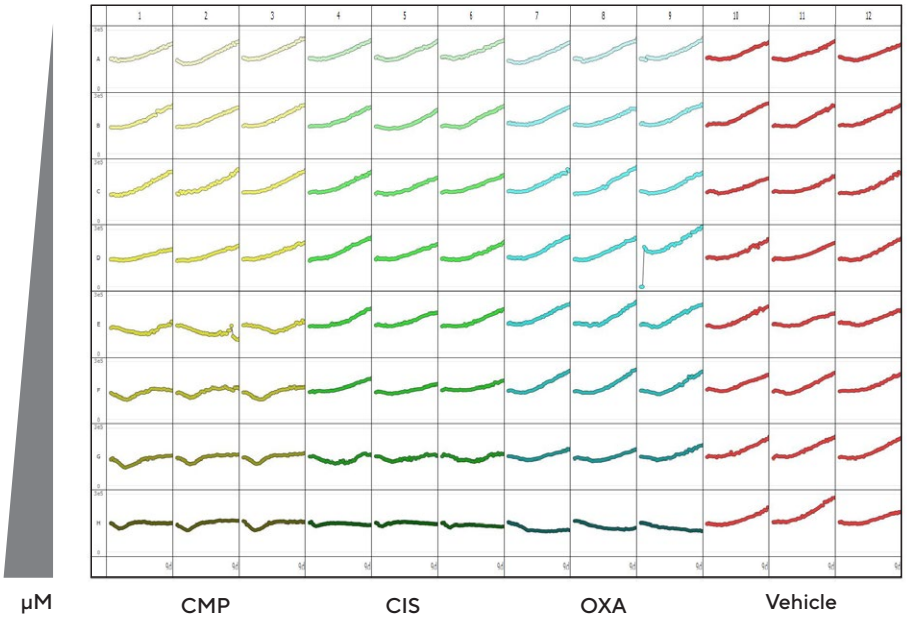


Figure 3: BF analysis enables accurate kinetic quantification of spheroids. The differential pharmacological effect of 1 µM CMP on growth of MDA-MB-231, HT-1080 and A549 cells in a 3D spheroid assay. Cells were grown in ULA round-bottom 96-well plates (2,500 cells per well) for 72 h and treatment with ±1 µM CMP followed. Segmented Brightfield images compare treated vs. untreated conditions at 240 h. Time courses illustrate the specific cell type-dependent kinetic profile of spheroid growth and shrinkage. The graphs display the Largest Brightfield Object Area (µm²) (y-axis) over the course of a 240 h assay (x-axis) at 6 h intervals. All images captured at 10X magnification. Each data point represents mean ±SEM, n = 4.

Demonstration of Pharmacological Utility

96-Well Spheroid Growth and Shrinkage Assay

To illustrate the amenability of our approach to drug toxicity testing, a pharmacological study was performed in the SKOV3 ovarian cancer cell line (Figure 4). Cells were grown in ULA round-bottom 96-well plates for 72 h, then treated either with the cytotoxic compound CMP, the apoptotic compound cisplatin (CIS), or the chemotherapeutic drug oxaliplatin (OXA). The effects on spheroid growth and shrinkage were quantified on the Incucyte® Live-Cell Analysis System by the evaluation of changes in spheroid area using the Largest Brightfield Object Area (µm²) metric. A concentration-dependent inhibitory growth effect was observed for all compounds, and illustrates how compound potencies can be directly compared within the same assay.



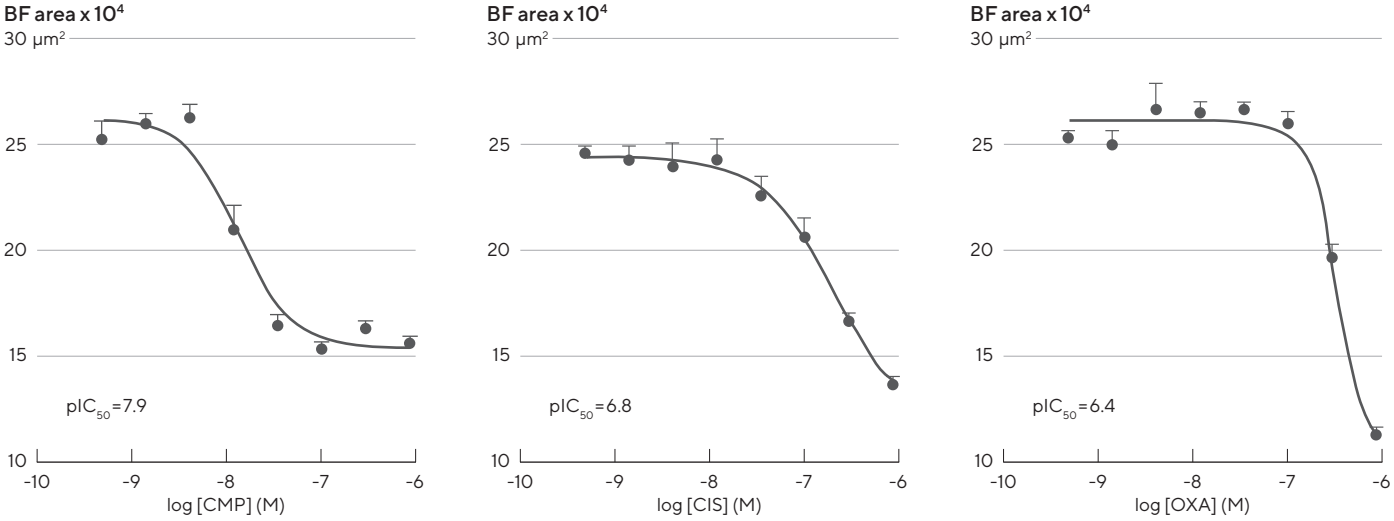


Figure 4: Effect of CMP, CIS and OXA on growth of SKOV3 cells in a 3D spheroid assay. SKOV3 cells were plated at a density of 5,000 cells/well and spheroid allowed to form (72 h). Cells were then treated with serial compound dilutions and kinetics of spheroid growth and shrinkage were obtained. Plate-view shows the individual well Largest Brightfield Object Area (µm²) over time. Concentration response curves represent the Largest Brightfield Object Area (µm²) at 204 h post-treatment. Data were collected over 240 h period at 6 h intervals. Each data point represents mean ±SEM, n = 8

Miniaturizing Spheroid Growth and Shrinkage Assay with Robust Intra- and Inter-Plate Reproducibility

To maximize data output and throughput, the 3D spheroid growth and shrinkage assay was miniaturized to 384-well format. A549, HT-1080 and SKOV3 cell types were tested at four seeding densities (Figure 5). The cells were plated in 384-well ULA round-bottom plates for 72 h, and spheroid Brightfield area was quantified on the Incucyte® Live-Cell Analysis System using the Largest Brightfield Object Area (µm²) metric. This data demonstrates the cell area dependence with seeding density and differential growth profile across the cell types.

Using the experiment performed above, both the intra- and inter-plate reproducibility was assessed. As shown, the spheroid size was seeding density dependent, and cell growth profiles were consistent with differential growth kinetics. Inter-plate variability analysis was performed at a seeding density of 2,500 cells per well. Reproducible Brightfield area metrics were obtained across plates for both vehicle and CMP-treated spheroids.

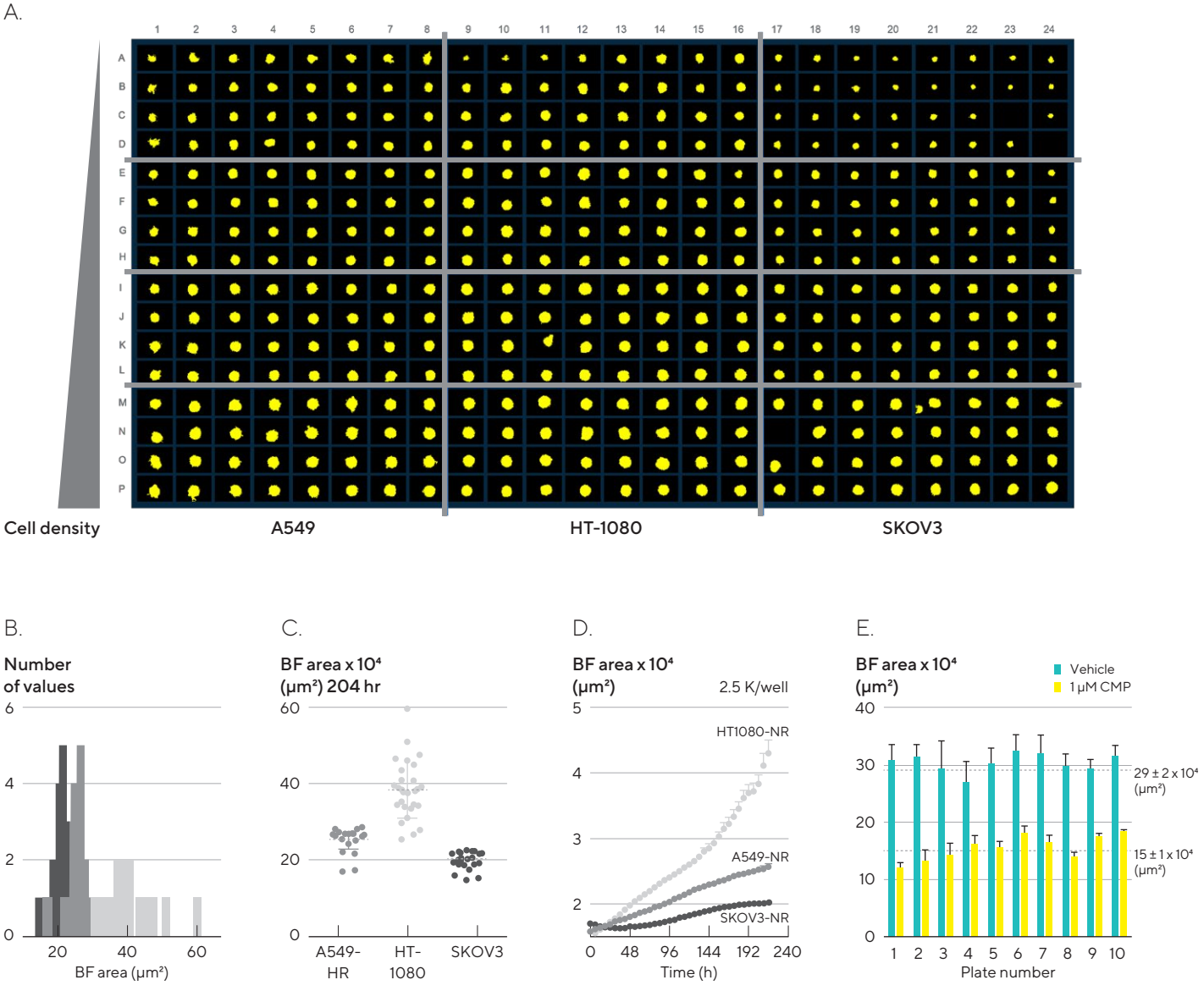


Figure 5: Miniaturizing spheroid growth and shrinkage assay for assay optimization. Comparison of temporal growth profiles of A549, HT-1080 and SKOV3 cells in a miniaturized 3D spheroid assay. (A) All cells seeded at a density ranging from 310–7,500 cells per well plated in a ULA round-bottom 384-well plate. Media was replenished 72 h post seeding. (A) Microplate overview image shows Brightfield segmentation mask at 204 h post-media replenishment. (B) Histogram compares the distribution frequency of the Brightfield Area (µm²) across all cell types plated at 2,500 cells/well at this time-point. (C) Variability plot analysis shows the largest Brightfield of individual wells at 204 h. (D) Time course plots represent the differential temporal profile of the Largest Brightfield Object Area metric (µm²) across the cell types. Data were collected over a 204 h period at 6 h intervals, all images captured at 10X magnification. Each data point represents mean ±SEM, n = 32. Reproducible BF area metrics across plates for vehicle- and CMP-treated spheroids (E) BF area metrics were reproducible across plates for both vehicle- and CMP-treated spheroids.

Investigation of Drug Mechanisms of Action with Viability and Toxicity Measurements

Expression of fluorescent proteins within the cells of a spheroid provide a readout of cell viability. Cytotoxic challenge will cause a loss of fluorescence signal, whereas spheroid growth leads to an increase in fluorescence. Masking of the Brightfield channel enables identification of the object of interest. Applying the "fluorescence within the Brightfield boundary" feature in the Incucyte® Spheroid Analysis Software Module allows determination of the mean fluorescence intensity of the pixels contained within the object of interest. The integrated fluorescence intensity within the Brightfield boundary provides an analysis suitable for determining both the growth and death of a spheroid from a single readout.

The effects of CMP on cell viability were studied in SKOV3 cells stably expressing a red fluorescent protein (Nuclight Red). The cells formed compact spheroids with identical properties to the wild-type cells based on Brightfield analysis (data not shown). Fluorescent measurements (integrated intensity within the Brightfield boundary) were determined post the 3 day formation stage (Figure 6 below). In vehicle treated spheroids, the fluorescence intensity increased proportionally to the increase in spheroid size. The addition of CMP (1 µM) induced a marked reduction in fluorescence, approaching background levels after 192 h. As described earlier, the fluorescence within a Brightfield boundary analysis negated the need to apply a fluorescence mask, and thus removed the impact of threshold settings.

The mechanisms of cell viability can also be explored by using Incucyte® Cell Health Reagents with spheroids (Cytotox Green | Red Dye, EssenBio Cat. No. 4632 | Cat. No. 4633 or Annexin V Green | Red Dye, EssenBio Cat. No. 4641 | Cat. No. 4642). Further, the inclusion of Incucyte® Annexin V Dyes and phosphatidylserine externalization markers, during formation or up to 96 h post-formation, enables the determination of apoptosis levels within the spheroid to be determined.

Incucyte® Cell Health Reagents were used to determine the mechanism of cell death after treatment with CMP (Figure 7). A549 cells (human lung carcinoma line) were seeded at 2,500 cells/well in the presence of Incucyte® Annexin V Green Dyes (EssenBio Cat. No. 4642) and formed compact spheroids. Spheroid size was monitored using Brightfield masking, which revealed a moderate increase in size over 10 d with vehicle-treatment. The addition of camptothecin (0.5 nM–1 µM) induced a concentration-dependent decrease in spheroid growth, abolishing a size increase at the highest concentration tested. Of note, little or no reduction in spheroid size was observed. Fluorescence analysis revealed a similar concentration-dependent effect with increases in the mean intensity within the Brightfield boundary, suggesting the induction of apoptosis. Plotting of concentration response curves for Brightfield and mean fluorescence yielded similar IC_{50} | EC_{50} values; 4.0 nM for the size analysis and 10.8 nM for the apoptotic readout.

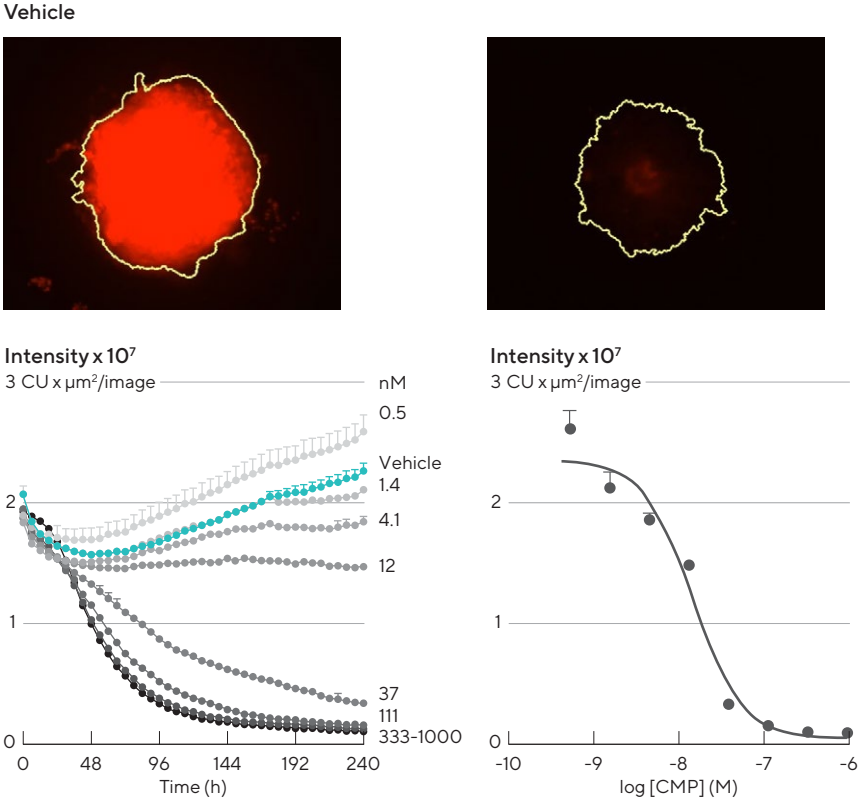


Figure 6: Analysis of spheroids expressing fluorescent proteins enables spheroid viability determination. Representative images taken at 240 h show a strong red fluorescent signal in a vehicle control spheroid, in contrast to a marked loss in red fluorescence in the CMP-treated spheroid. The yellow boundary in the images represents the Brightfield mask outline. Monitoring the integrated intensity from within the Brightfield boundary highlights a gradual increase in fluorescence under vehicle control conditions (gray symbols) corresponding to the growth of the spheroid. Upon treatment with CMP, a concentration-dependent reduction in integrated fluorescence is observed, with abolishment of fluorescence with the highest concentration tested after 240 h.

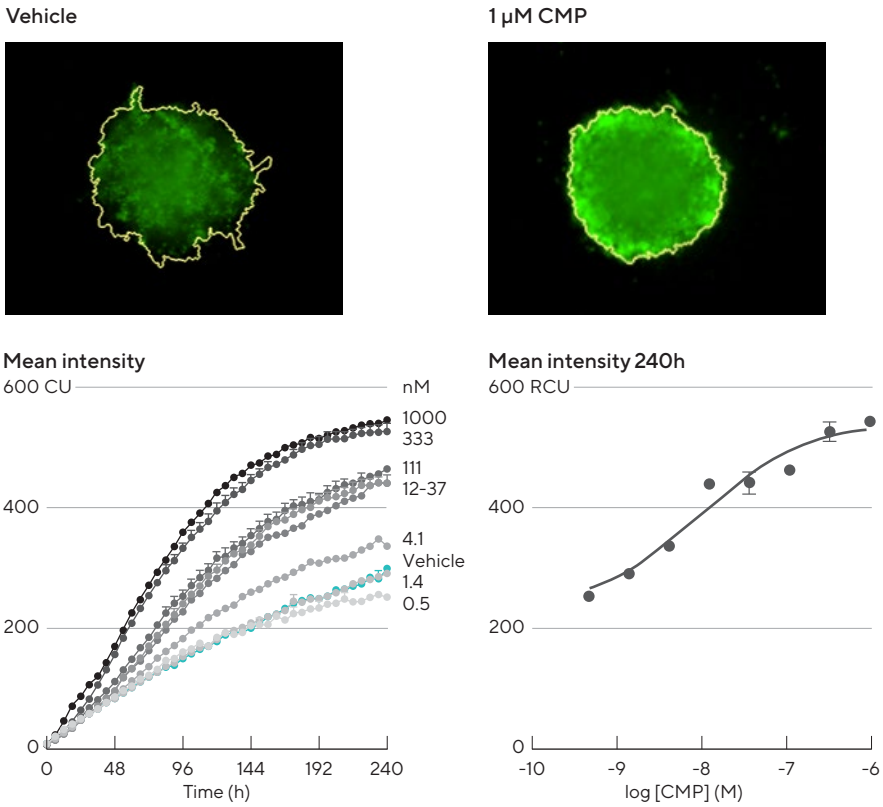


Figure 7: Effect of camptothecin (CMP) on A549 cells reported by Annexin V Green Dye in a 3D spheroid assay. A549 cells were seeded at a density of 2,500 cells/well in ULA round bottom plates and spheroids were formed for 96 h. Spheroids were treated with CMP (0.5 nM–1 µM) or vehicle (0.1% DMSO), and apoptosis was reported using Annexin V Green.

Finally, both Brightfield and Cell Health readouts may be combined in the single spheroid assay to determine the mechanism of drug action. The Brightfield analysis, although powerful as it requires no labeling, does not readily distinguish between cytotoxic and cytostatic agents due to the strong Brightfield signature of non-viable spheroids. In contrast, determining the viability of the spheroid has the potential to discriminate between cytotoxic and cytostatic agents, with the latter expected to yield marked effects on spheroid size while inducing little cytotoxicity.

To demonstrate pharmacology applicability and versatility, the effects of the cytotoxic agent camptothecin and cytostatic agent cycloheximide were evaluated on SKOV3 spheroids formed in the presence of the cytotoxicity marker Incucyte® Cytotox Green Dye (Figure 8). Both agents induced concentration-dependent attenuation of spheroid growth, yielding IC_{50} values of 24.6 nM for CMP and 1.5 µM for CHX. Interestingly, CMP yielded a marked increase in the mean fluorescence, suggesting a cytotoxic mechanism. The CMP EC_{50} value for cytotoxicity was 51.4 nM, similar to that returned for the Brightfield (size) determination. By contrast, CHX only yielded a notable increase in fluorescence at the highest concentration tested (10 µM), not allowing an EC_{50} to be determined. The clear separation between the size and cytotoxicity readouts supports the cytostatic mechanism of CHX.

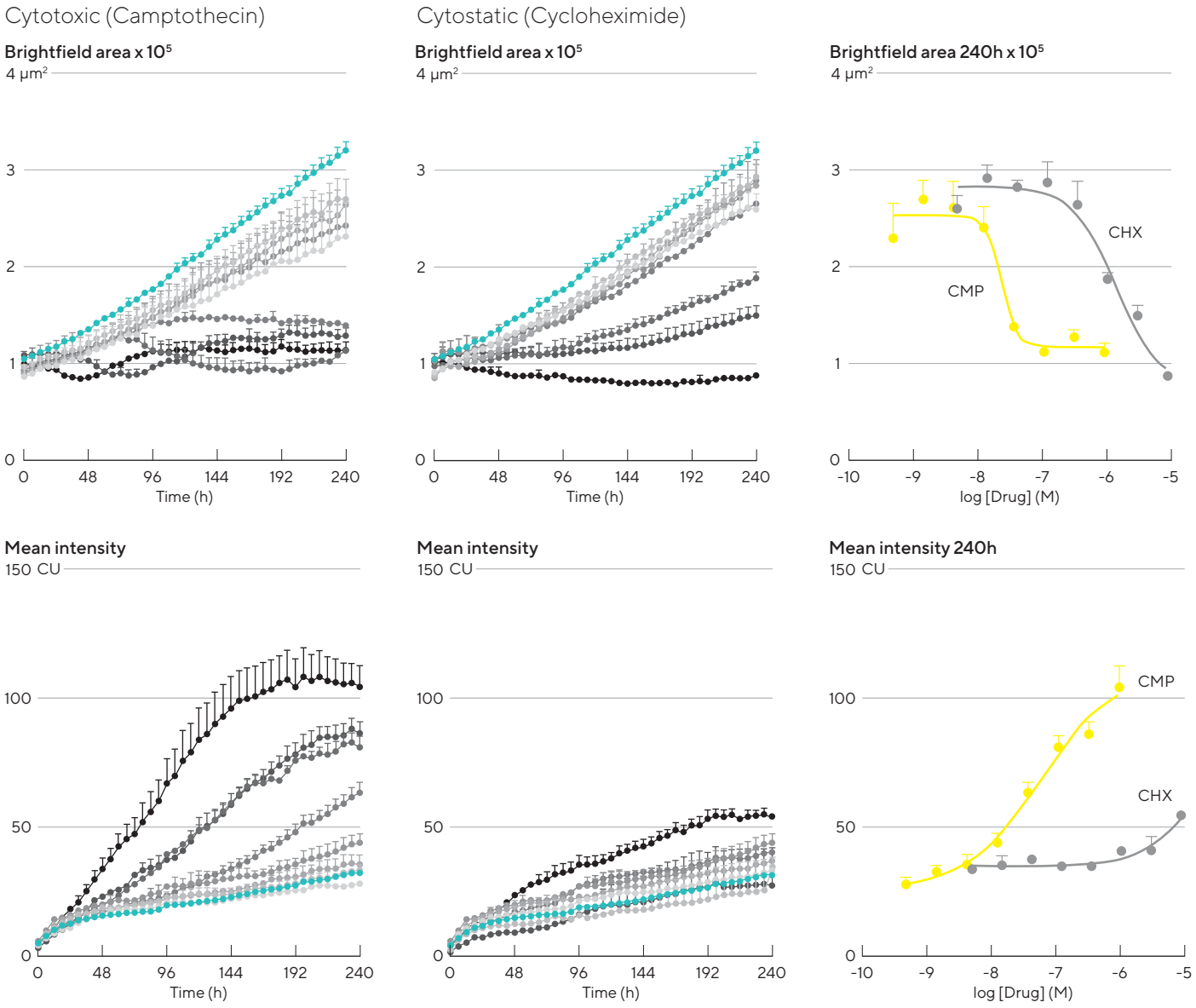


Figure 8: Cytotoxic and cytostatic mechanisms of action can be differentiated by measuring spheroid size and viability. SKOV3 cells were plated at a density of 2,500 cells/well and spheroid allowed to form (96 h). Spheroids were treated with increasing concentrations of camptothecin (0.5 nM–1 µM) or cycloheximide (1.4 nM–10 µM) in the presence of Cytotox Green Dye (25 nM). Images were taken every 6 h for 10 d. Time courses show change in Brightfield area (top row) or fluorescent response (bottom row) over time. CRCs show the different profiles of cytotoxic and cytostatic mechanisms.

Conclusions

In this application note, we have demonstrated that the Incucyte® Live-Cell Analysis System enables the study of 3D single spheroid analysis in real time, and that this application can be highly useful for scientific investigation, as well as downstream pharmacological analysis. Brightfield, in combination with phase contrast imaging, allows for label free study of 3D spheroid morphology, growth and shrinkage in 96- and 384-assay formats. Incucyte® HD phase images facilitate comprehensive visualization of spheroid morphological features (shape, size) and intercellular compaction (loose aggregates vs. compact spheroids) characteristic for each cell type. BF provides the means for objective spheroid kinetic quantification and cell dependent growth rate profile assessment for diverse type of spheroids.

Furthermore, we have also exemplified that this platform's kinetic analyses, in conjunction with Incucyte® Cell Health Reagents or fluorescent probes, enabled the study of spheroid viability in real time. Combining viability determination with the established benefits of Brightfield size analysis and phase contrast morphological insights, facilitates a comprehensive spheroid analysis solution in one convenient platform.

With no need for predefined end-point selection, a highly consistent Brightfield segmentation, reproducible well-to-well kinetic data, and robust intra- and inter-plate reproducibility, Incucyte® Live-Cell Imaging and Analysis demonstrates amenability to pharmaceutical investigations. The user-friendly platform enables operators to assess real-time information, perform analysis, and generate publication ready results, quickly and efficiently. The use of Incucyte® Single Spheroid Assays, Incucyte® Reagents, and the Incucyte® Spheroid Analysis Software Module provides a unique workflow niche, in the form of real-time kinetic information, to guide both basic science research as well as drug discovery and development.

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