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Application Note

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Validation of Real-Time, Live-Cell Assays for 3D Multi-Spheroids Formed on Bio-Matrices

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Introduction

The use of multi-cell tumor spheroids as a model for oncology research has rapidly expanded due to a greater appreciation for the influence of the tumor microenvironment, as well as spatial interactions between cells. 3D microtissues and organoids may provide more physiologically relevant observations as compared to 2D monolayer cell models, where cells are grown on non-biological surfaces under hyper-nourished conditions that may confound drug efficacy or metabolic studies.[1,2] As 3D spheroid protocols become more accessible, the experimental models have become more complex with greater translational potential, often incorporating additional elements that better replicate the heterogeneity of the cellular microenvironment.

Of particular importance for improved physiological relevance is the incorporation of the extracellular matrices (ECM) and other cell types found within the microenvironment. Multi-cell spheroids can deposit extracellular matrix (ECM) components, similar to a tumor *in vivo*, and the inclusion of ECM scaffolds (such as Matrigel®) enables the cell-ECM interface to be probed in greater detail. Spheroid models also have a greater level of cell-to-cell contact to better replicate structures and study the effect of cellular interactions on drug treatment.[3] As the spheroids grow, they also begin to display internal layered structures, similar to that within tumors, including growth kinetics and nutrient gradients that could influence drug resistance.[4] Additionally, they produce gene expression profiles that are more physiologically relevant.[1]

Despite these strengths, current methods for assessing the growth and shrinkage of 3D tumor spheroids cultured with ECM are limited by one or more of the following: 1. assay workflows that are time-consuming, expensive or laborious requiring trained operators for analysis; 2. a requirement for labeled cells with fluorescent probes that may perturb biological responses and may not be suitable for primary tissue; 3. single time point readouts that do not report the full time course of growth, nor provide kinetic visualization of spheroid morphology; 4. removal of cells from physiological conditions for image acquisition; 5. indirect readouts (e.g., ATP) that may overlook valuable morphological insight and/or misreport cell growth. There is a need for higher throughput, easily accessible monitoring and analysis methods that impart minimal disruption of cells, and can be performed in real time and long-term for temporal analysis of cellular growth, and which can also do so in the presence of ECM components in order to provide more physiological relevant information.

Assay Principle

This application note describes the use of the Incucyte® Live-Cell Analysis system and Incucyte® 3D Multi-Tumor Spheroid Assays to study the growth of 3D spheroids label-free or with non-perturbing reagents in real time, capturing data that may be missed by single time point methods. The enhanced depth of focus Brightfield (DF® Brightfield) image acquisition enables long term imaging of multiple tumor spheroids grown on a bed of extracellular matrix (Matrigel®). This enhanced image acquisition results in Brightfield images with high contrast, which can be readily masked using built-in Incucyte® processing definitions. Brightfield object size, count and eccentricity are automatically plotted over time and deliver a wealth of information on spheroid formation, health and growth rates. In combination with Incucyte® Cell Health reagents or cells expressing fluorescent proteins, the ability to quantify fluorescence within the Brightfield mask provides objective data on spheroid viability, death and levels of apoptosis. The ability to multiplex reagents with fluorescently-labeled cell lines provides further information on cellular mechanisms of action. Thousands of images may be acquired, analyzed, and graphed, with the capability to run up to six 96-well plates in parallel for increased throughput.

Materials and Methods

Quick Guide

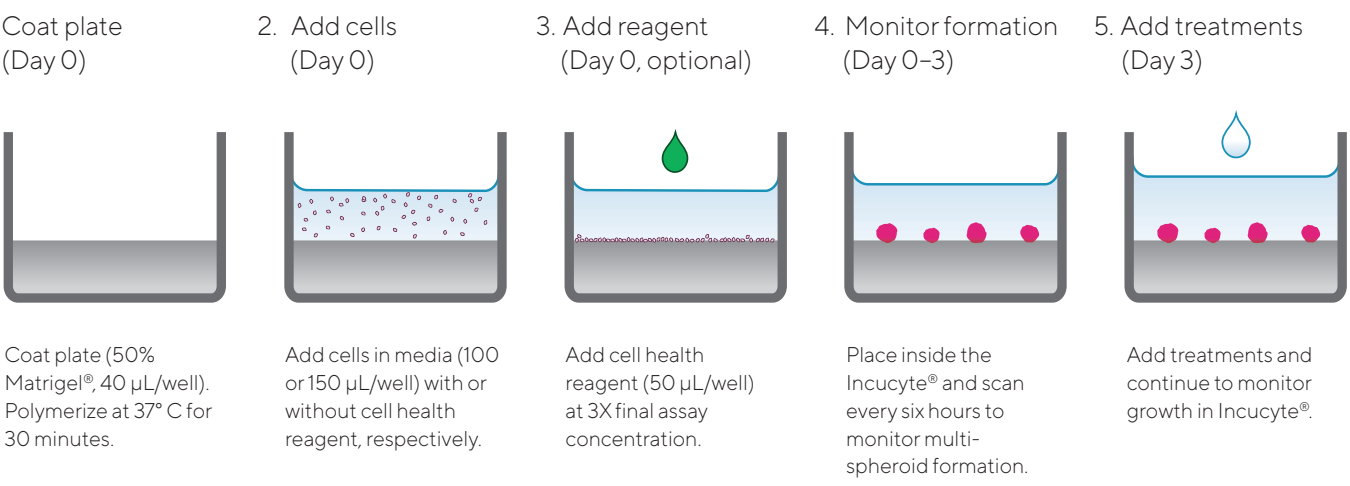


Figure 1: Assay Workflow

1. A flat-bottom 96-well plate is coated with a layer of Matrigel® (Corning) (40 µL /well, diluted in serum-free media to a minimum concentration of 4.5 mg/mL) which is then polymerized at 37° C for 30 min.
2. Cells of interest are harvested, counted and seeded onto plates at desired densities (150 or 100 µL/well).
   a. If required, cell health reagents are added in 50 µL/well.
   b. The total volume of media at this stage should be 150 µL/well.
3. Spheroid formation is monitored for 72 h with DF-Brightfield and HD phase-contrast image acquisition at 10X magnification every 6 h using Incucyte®.
4. Compounds are added (50 µL/well at 4X final assay concentration- FAC; or, if using Cell Health reagents, 15 µL/well at 11X FAC).
5. The spheroid growth and shrinkage assay is initiated and monitored in the Incucyte® (6 h repeat scanning, up to 2 weeks). Tumor size is reported in real time based on DF-Brightfield image analysis.

All cell culture reagents were obtained from Life Technologies unless otherwise noted. MDA-MB-231 (ATCC) and SKOV3 (EACC) cultures were stably transfected with Incucyte® Nuclight Red Lentivirus Reagent (EF1 Alpha Promoter, Puromycin selection, Cat. No. 4625, prepared per Essen BioScience protocol). Incucyte® Nuclight Red cell lines (A549-NR, Essen BioScience Cat. No. 4491; MCF-7-NR, Essen BioScience Cat. No. 4524) as well as the prepared SKOV3-NR and MDA-MB-231-NR were grown to confluence in 75 cm^2 tissue culture treated flasks and seeded into 96-well plates (Corning #3595) coated with a base layer of 40 µL/well Matrigel® such that by 72 h spheroids formed with desired size. All cells were cultured in F-12K medium supplemented with 10% FBS, 1% Pen | Strep, 1% Glutamax and 0.5 µg/mL puromycin. Spheroid formation was monitored in an Incucyte® over a 72 h period at 6 h intervals. Cell viability of MDA-MB-231 cells was determined using Incucyte® Cytotox Green (Essen BioScience Cat. No. 4633). Apoptosis of A549 spheroids was evaluated by quantification of phosphatidylserine (PS) externalization (Incucyte® Annexin V Green, Essen BioScience Cat. No. 4642).

Cell-Type Dependent Label-Free Kinetic Spheroid Growth Profiles

The size of tumor spheroids was measured over time using an automated software algorithm that masked the Brightfield object in the field of view. Changes in the size of A549, MCF-7 and MDA-MB-231 tumor spheroids were monitored over time, in the absence and presence of the cytotoxic compound camptothecin (CMP; Figure 2). Cell type dependent kinetic growth profiles were observed for the cells tested. For all cell types, the control spheroids increased markedly over the 7-day period (2.4–3.7 fold) with the largest size increase observed with MCF-7 spheroids.

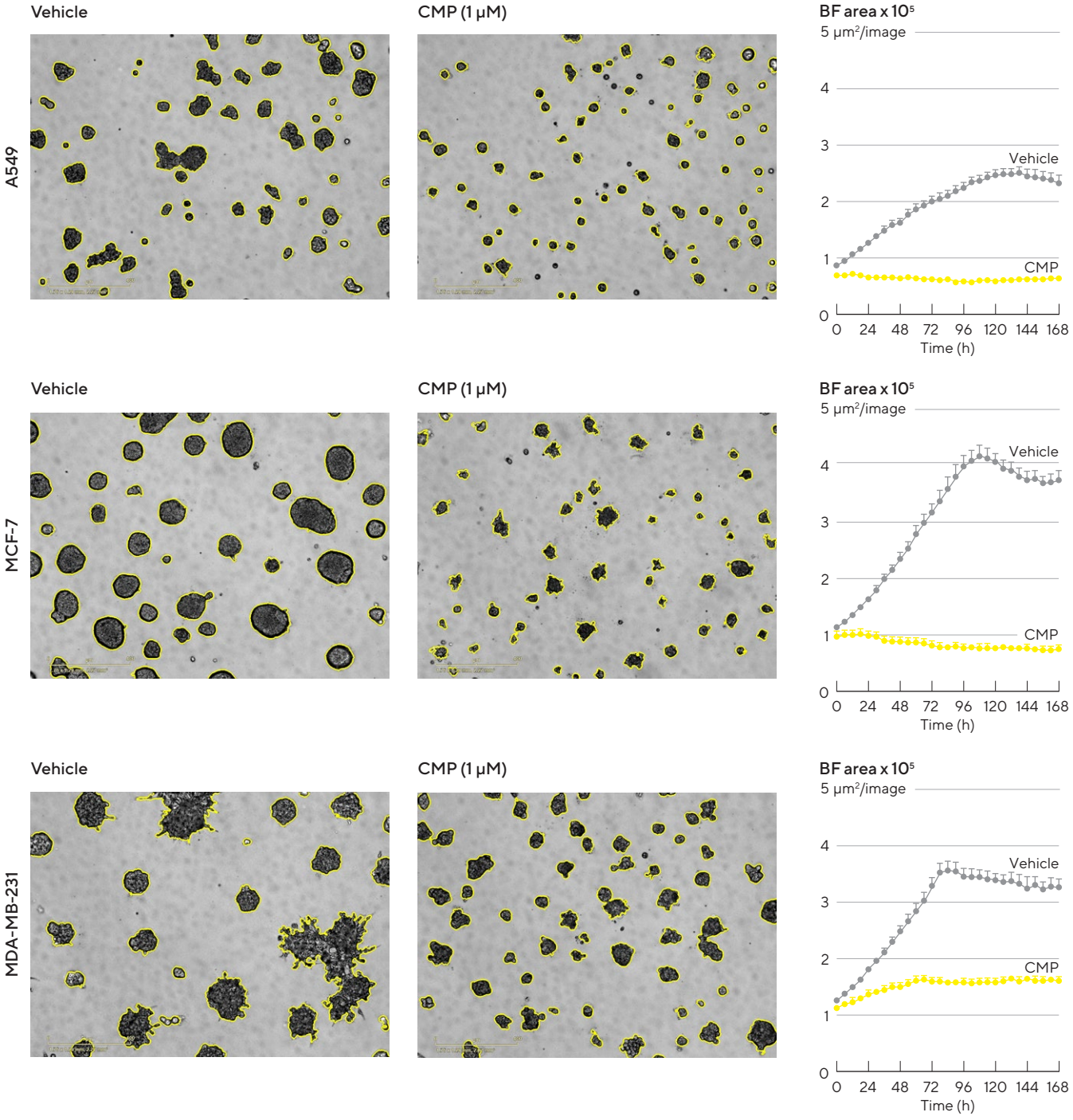
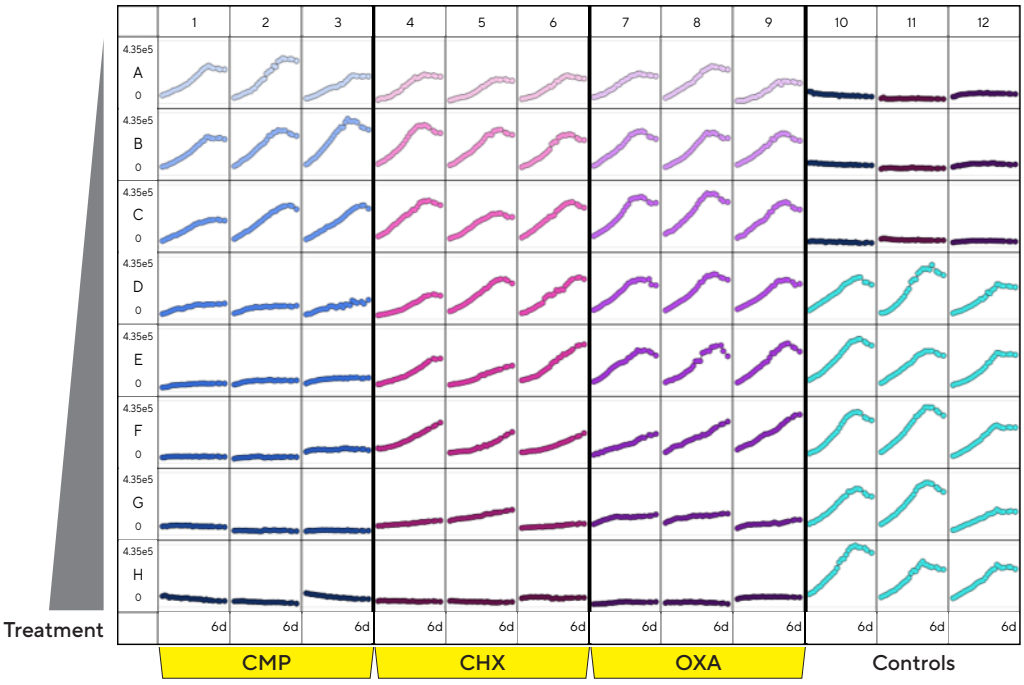


Figure 2: Quantification of cell-type dependent kinetic growth profiles of A549, MCF-7 and MDA-MB-231 spheroids using real time analysis. Cells were seeded in flat bottom 96-well plates (2,000 cells/well) on a bed of Matrigel® and spheroids allowed to form (72 h). Spheroids were treated with either vehicle (0.1% DMSO) or CMP (1 µM). Segmented (yellow outline) DF-Brightfield images compare vehicle or CMP treated conditions at 168 h. Morphological differences are shown between round (A549, MCF-7) and stellate (MDA-MB-231) spheroids. Time courses show the individual well Total Brightfield Object Area (µm²) (y-axis) over 168 h and illustrate cell type specific kinetic growth profiles. Data were collected over 168 h period at 6 h intervals. All images captured at 10X magnification. Each data point represents mean ± SEM, n=6 wells.

96-Well Spheroid Growth and Shrinkage Assay for Pharmacological Analysis

To illustrate the amenability of this approach to drug toxicity testing, a pharmacological study was performed in MCF-7 breast cancer cells. Spheroids were formed over 72 h in flat bottom 96-well plates coated with a layer of Matrigel® prior to treatment with camptothecin (CMP), cycloheximide (CHX) or the chemotherapeutic drug oxaliplatin (OXA) (Figure 3). All compounds produced a concentration-dependent inhibition of spheroid growth, illustrating how compound potencies can be easily compared within the same assay.



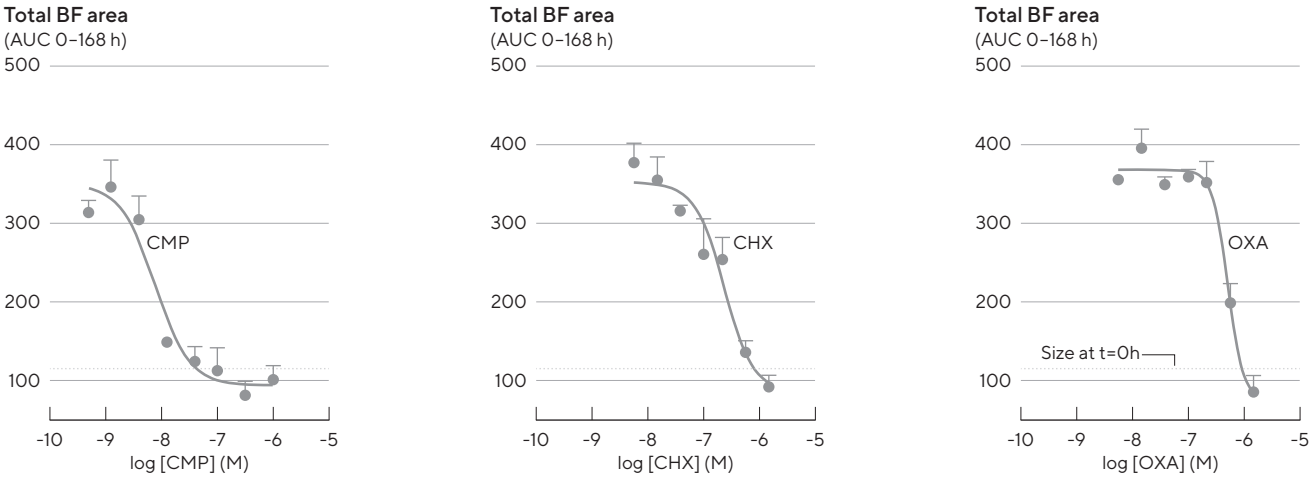


Figure 3: Incucyte® enables comparison testing of drug toxicities on growth of MCF-7 cells over time in a single microplate 3D spheroid assay. MCF-7 cells were seeded in flat bottom 96-well plates (1,000 cells/well) on a bed of Matrigel® and spheroids allowed to form (72 h). Cells were then treated with serial dilutions of CMP, CHX, or OXA and spheroid growth kinetics obtained. The plate view shows the individual well Total Brightfield Object Area (μm²) (y-axis) over time (6 days) (x-axis). Concentration response curves (CRCs) represent the area under curve (AUC) of the Total Brightfield Area time course (μm²) from 0–168 h post-treatment. Data were collected over 168 h period at 6 h intervals. Each data point represents mean ± SEM, n=3 wells.

Cell Death Assay

The introduction of Incucyte® Cell Health reagents to this assay enables the monitoring of cell death. Cell death can be determined using Incucyte® Cytotox reagents that become fluorescent and bind to DNA once they cross a compromised cell membrane (Incucyte® Cytotox Green | Red Reagent, Essen BioScience Cat. No. 4633 | 4632), and apoptosis can be evaluated by quantification of phosphatidylserine (PS) externalization (Incucyte® Annexin V Green | Red Reagent, Essen BioScience Cat. No. 4642 | 4641) or activation of Caspase 3/7 (Incucyte® Caspase 3/7 Green Reagent, Essen BioScience Cat. No. 4440).

MDA-MB-231 cells, seeded in either the absence or presence of Incucyte® Cytotox Green reagent (250 nM), formed spheroids of comparable size and morphology (data not shown). Fluorescence analysis from within the Brightfield boundary of the vehicle controls revealed little or no increase in fluorescence until 192 h, after which a small rise was observed, suggesting some cell death at later time points. CMP induced a marked time-dependent increase in fluorescence intensity indicating loss of spheroid viability (Figure 4).

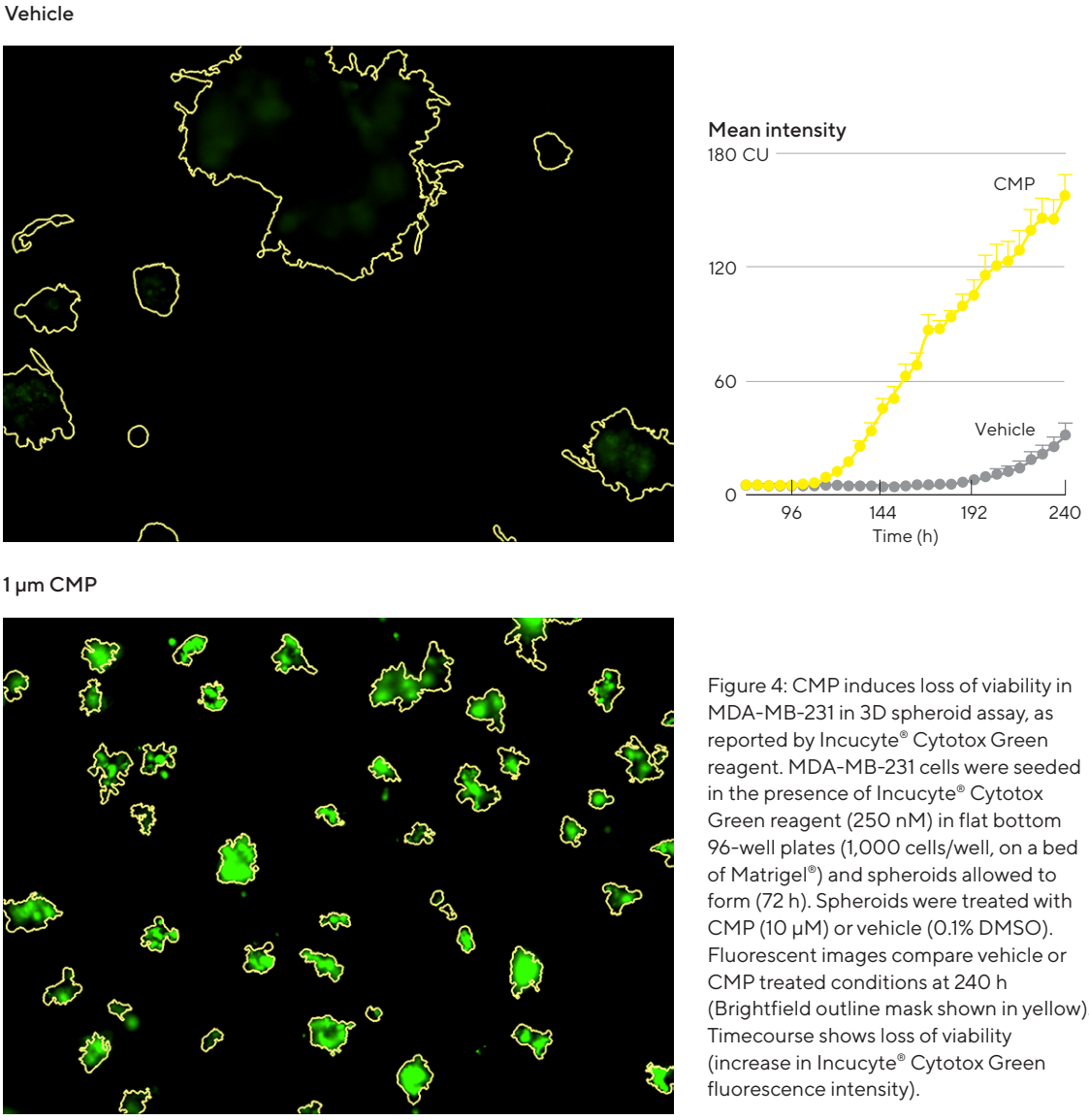


Figure 4: CMP induces loss of viability in MDA-MB-231 in 3D spheroid assay, as reported by Incucyte® Cytotox Green reagent. MDA-MB-231 cells were seeded in the presence of Incucyte® Cytotox Green reagent (250 nM) in flat bottom 96-well plates (1,000 cells/well, on a bed of Matrigel®) and spheroids allowed to form (72 h). Spheroids were treated with CMP (10 µM) or vehicle (0.1% DMSO). Fluorescent images compare vehicle or CMP treated conditions at 240 h (Brightfield outline mask shown in yellow). Timecourse shows loss of viability (increase in Incucyte® Cytotox Green fluorescence intensity).

Determining Mechanism of Drug Action Using Spheroid Growth and Viability

Combining the size and levels of cytotoxicity | apoptosis in spheroids can yield important information on the mechanism of action of a compound. The Brightfield analysis, although powerful as it requires no labeling, does not fully 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 cell death.

The level of apoptosis in spheroids can be determined using Incucyte® Annexin V Apoptosis reagent (a probe for phosphatidylserine externalization) and quantified using the mean fluorescence within the Brightfield boundary.

The effects of the cytotoxic agent camptothecin (CMP) and cytostatic agent cycloheximide (CHX) were evaluated on A549 spheroids formed in the presence of Incucyte® Annexin V Green (Figure 5). Both agents induced concentration-dependent attenuation of spheroid growth, with CMP shrinking the spheroid to a size less than that observed at time of treatment. Analysis of Brightfield size returned IC^{50} values of 42 nM for CMP and 0.22 µM for CHX. Interestingly, CMP generated a marked increase in the mean fluorescence, suggesting a cytotoxic mechanism. The CMP EC^{50} value for apoptosis induction was 0.30 µM, similar to the value obtained from the size analysis. By contrast, CHX did not cause an increase in mean fluorescence, therefore, not allowing an EC^{50} to be determined. The clear separation between the size and cytotoxicity readouts supports the cytostatic mechanism of CHX. Long-term continual live-cell analysis, maintained in physiologically relevant conditions, allows observation of concentration response curves with minimal disturbance to cellular biology, allowing determination of meaningful EC^{50} values.

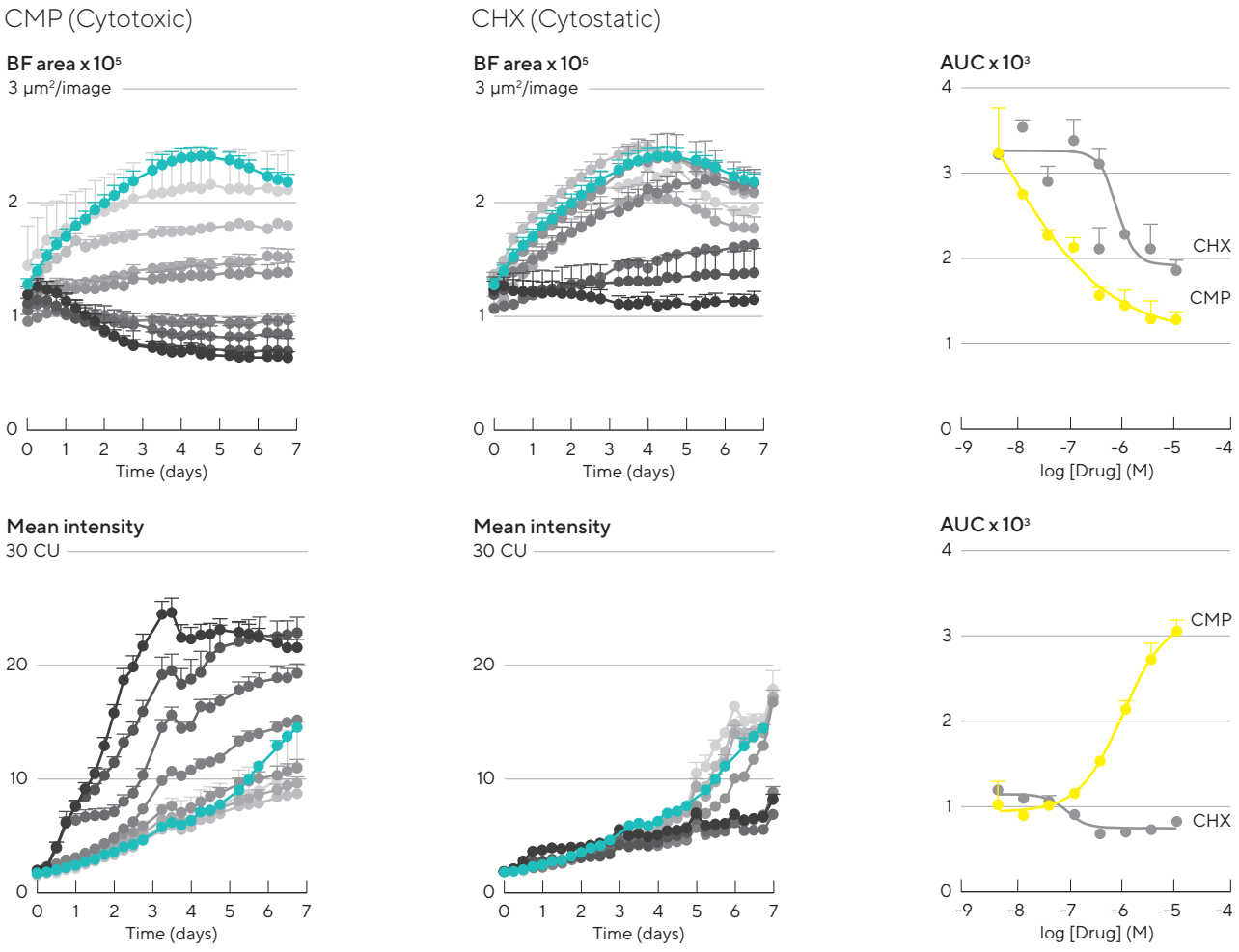


Figure 5: Cytotoxic and cytostatic mechanisms of action can be differentiated by measuring spheroid size and apoptosis over time. A549 cells were seeded in the presence of Incucyte® Annexin V Green reagent (1%) in flat bottom 96-well plates (2,000 cells/well, on a bed of Matrigel®) and spheroids allowed to form (96 h). Spheroids were then treated with increasing concentrations of CMP (left column, 4 nM–10 µM) or CHX (right column, 4 nM–10 µM). Time courses show change in size (Brightfield Area) or apoptosis (Incucyte® Annexin V fluorescence intensity) over time. CRCs show the different profiles of cytotoxic and cytostatic mechanisms.

Monitoring Spheroid Response to Cytotoxic Challenge Over Time with Fluorescent Proteins

Expression of fluorescent proteins within the cells of a spheroid provide a readout of cell viability, where spheroid growth leads to an increase in fluorescence, and cytotoxic challenge will cause a loss of fluorescence signal. Masking of the Brightfield channel enables identification of the objects of interest. Applying the 'fluorescence within the Brightfield boundary' feature in the Incucyte® S3 Spheroid Analysis software module allows determination of fluorescence intensity of the pixels contained within the object of interest. Using the integrated fluorescence intensity within the Brightfield boundary provides an analysis suitable for determining both growth and death of labeled spheroids from a single readout.

MCF-7 cells stably expressing a red fluorescent protein (Incucyte® Nuclight Red) 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). In vehicle treated spheroids, the fluorescence intensity increased proportional to the increase in spheroid size. Addition of CMP (0.4 nM–1 µM) induced a marked reduction in fluorescence, approaching background levels at 144 h. As described earlier, the fluorescence within a Brightfield boundary analysis negates the need to apply a fluorescence mask, and thus, removes the impact of threshold settings.

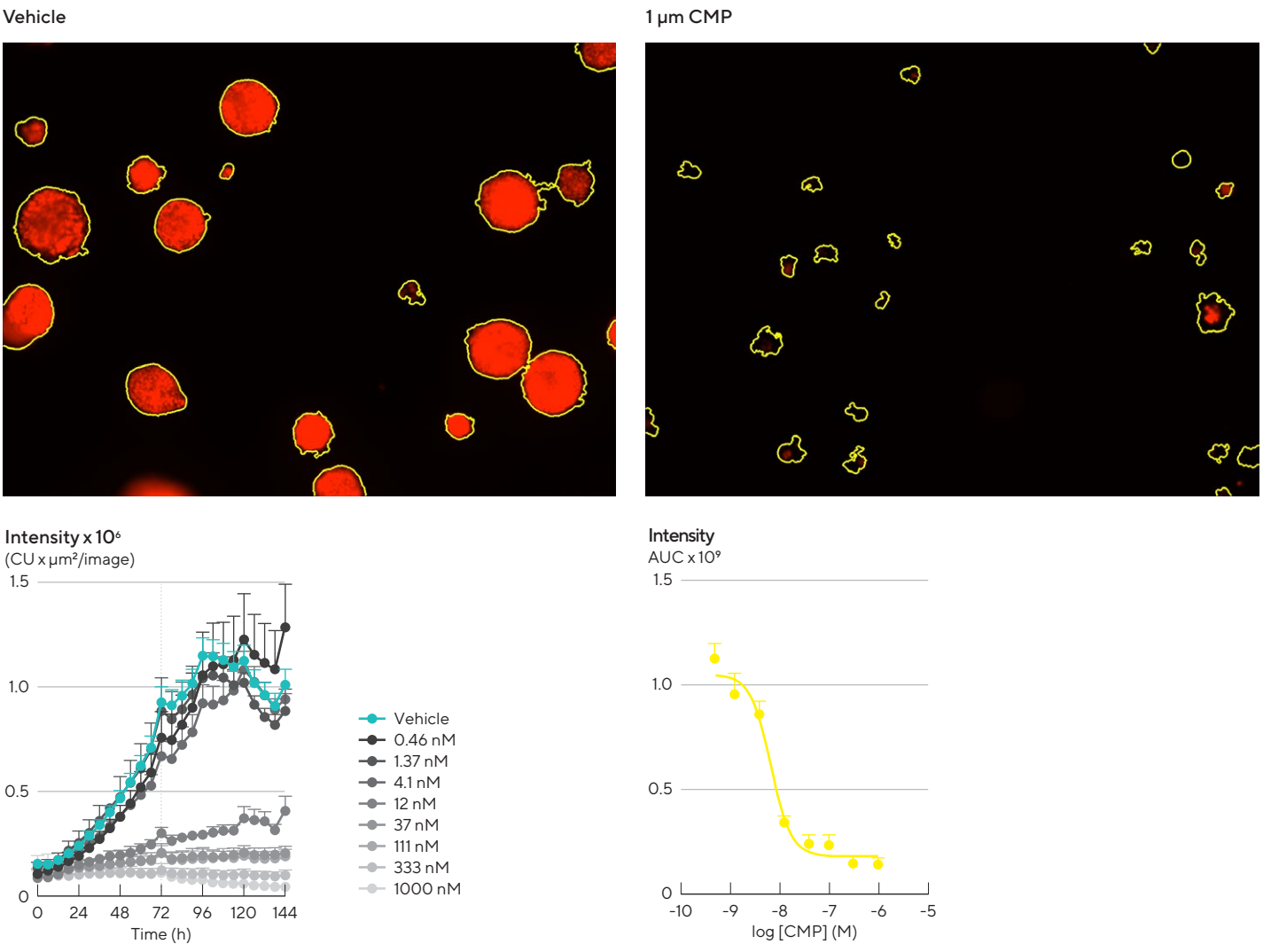


Figure 6: Analysis of spheroids expressing fluorescent proteins enables determination of concentration-dependent spheroid viability over time. MCF-7 cells were seeded in flat bottom 96-well plates (1,000 cells/well) on a bed of Matrigel® and spheroids allowed to form (72 h). Representative images taken at 144 h show a strong red fluorescent signal in a vehicle (0.1% DMSO) treated spheroids and a marked loss of red fluorescence in CMP treated spheroids. The yellow boundary in the images represents the Brightfield mask outline. Monitoring the integrated intensity from within the Brightfield boundary highlights increasing fluorescence under vehicle control conditions corresponding to the growth of the spheroid. Upon treatment with CMP (0.4 nM–1 µM), a concentration-dependent reduction in integrated fluorescence is observed, with abolishment of fluorescence with the highest concentration tested after 144 h.

Conclusions

In this application note, we demonstrate that the Incucyte® Live-Cell Analysis System, in conjunction with the Incucyte® Spheroid Software Module and validated Incucyte® Cell Health Reagents, enables the analysis of 3D multi-spheroids over time, and is applicable to drug testing. We have shown here that:

- Live-cell imaging revealed morphological differences between A549, MCF-7 and MDA-MB-231 cells (round vs. stellate).
- The system generated cell-type dependent kinetic growth profiles for different cell types.
- Comparison testing of drug toxicities of camptothecin (CMP), cycloheximide (CHX), and oxaliplatin (OXA) were easily performed within one assay.
- The temporal loss of cell viability in response to CMP treatment was captured using the Cytotox Green reagent.
- This assay was able to distinguish between cytotoxic and cytostatic mechanisms of drug action in the response to camptothecin (CMP) and cycloheximide (CHX) respectively.
- Concentration dependent changes in spheroid viability were monitored over time with the fluorescent protein, Incucyte® Nuclight Red, in response to cytotoxic challenge with CMP.

Several features of the Incucyte® Live-Cell Analysis System are particularly advantageous for monitoring and objectively quantifying 3D spheroid kinetics and profiling growth rates for diverse spheroid types. The DF-Brightfield imaging allows for label-free study of 3D spheroid morphology, growth and shrinkage in 96-well assay formats for enhanced throughput. There is no need to select predefined end points, and the system produces a consistent Brightfield segmentation with reproducible well-to-well kinetic data. This system's automated image acquisition, combined with user-friendly analysis tools and lab tested protocols, allows non-expert users to quickly generate reproducible data, perform analysis, and generate publication ready graphics.

Taken together, the Incucyte® Live-Cell Analysis System, Spheroid Software Module and reagents provides a unique and efficient technical platform that can be incorporated into existing workflows. This system provides kinetic, non-perturbing physiological characterization of spheroid growth and viability, and is amenable to pharmacological discovery and development.

References

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Tumor Microenvironment
Salo T, *et al.* **Organotypic three-dimensional assays based on human leiomyoma-derived matrices.** *Philos Trans R Soc Lond B Biol Sci,* Jan 5; 373(1737) (2018)

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Product Inquiry

Online Product Inquiry

Reagent Ordering Information

Product	Cat. No.	Size
Incucyte® S3 Spheroid Software Module	9600-0019	1 module
Incucyte® Nuclight Green Lentivirus (EF-1a Promoter, Puro selection) Nuclear Labeling Reagent	4624	1 vial (0.2 mL)
Incucyte® Nuclight Red Lentivirus (EF-1a Promoter, Puro selection) Nuclear Labeling Reagent	4625	1 vial (0.2 mL)
Incucyte® CytoLight Green Lentivirus (EF-1a Promoter, Puro selection) Cytoplasmic Labeling Reagent	4481	1 vial (0.6 mL)
Incucyte® CytoLight Red Lentivirus (EF-1a Promoter, Puro selection) Cytoplasmic Labeling Reagent	4482	1 vial (0.6 mL)
Incucyte® Caspase-3/7 Green Apoptosis Reagent	4440	One vial (20 µL)
Incucyte® Annexin V Red Reagent	4641	One vial (100 tests)
Incucyte® Annexin V Green Reagent	4642	One vial (100 tests)
Incucyte® Cytotox Red Reagent	4632	Five vials (5 µL)
Incucyte® Cytotox Green Reagent	4633	Five vials (5 µL)

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