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Application Note

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Real-Time Live-Cell Analysis of Multi-Spheroid, Co-Cultured, 3D Tumor Assays

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Introduction

A growing body of evidence suggests studies involving microtissues and organoids provide more predictive and translational observations as compared to 2D monolayer cell models[1,2]. Additionally, the use of multicellular tumor spheroids for oncology and immuno-oncology research is increasing. *In vitro* models that attempt to recapitulate the tumor microenvironment may incorporate an extracellular matrix (ECM) and additional cell types, such as stromal, endothelial and immune cells, allowing researchers to assess functionality of drugs, evaluate chemoresistance and immune-tumor cell interactions.

Current methods for assessing the growth and shrinkage of tumor spheroids are often limited by time-consuming, expensive and/or laborious assay workflows. These may include fluorescent probes that perturb the biology, an end-point analysis that might miss insightful temporal information, or indirect biochemical readouts that overlook valuable morphological insight.

The Incucyte® Live-Cell Analysis System is a fully automated phase contrast and two-color fluorescence image acquisition and analysis platform that resides within a standard cell culture incubator for optimal cell viability and maintenance of physiological relevance. The instrument is designed to scan tissue culture plates and flasks repeatedly over pre-determined time intervals, enabling users to continuously monitor cultures and generate quantifiable, kinetic information. The Incucyte® Spheroid software module acts as a fast, flexible and powerful control hub for continuous live-cell analysis including image acquisition, processing, and data visualization. Reagents include non-perturbing, cell labeling, nuclear-targeted GFP (green fluorescent protein), and RFP (red fluorescent protein) for cell quantification.

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 tumor multi-spheroids in co-culture with either fibroblasts or immune cells, 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 superior image acquisition results in Brightfield images with high contrast that can be readily masked using built-in Incucyte® processing definitions. Brightfield object size, count and eccentricity are automatically plotted over time to deliver a wealth of information on spheroid formation and growth rates. 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.

Here we present validation methods and data that demonstrate the ability of the Incucyte® Live-Cell Analysis System to kinetically visualize and quantify the impact of stromal cells on tumor multi-spheroid morphology and sensitivity to chemotherapeutic agents, as well as to assess immune cell-mediated toxicity within tumor multi-spheroids.

Material and Methods

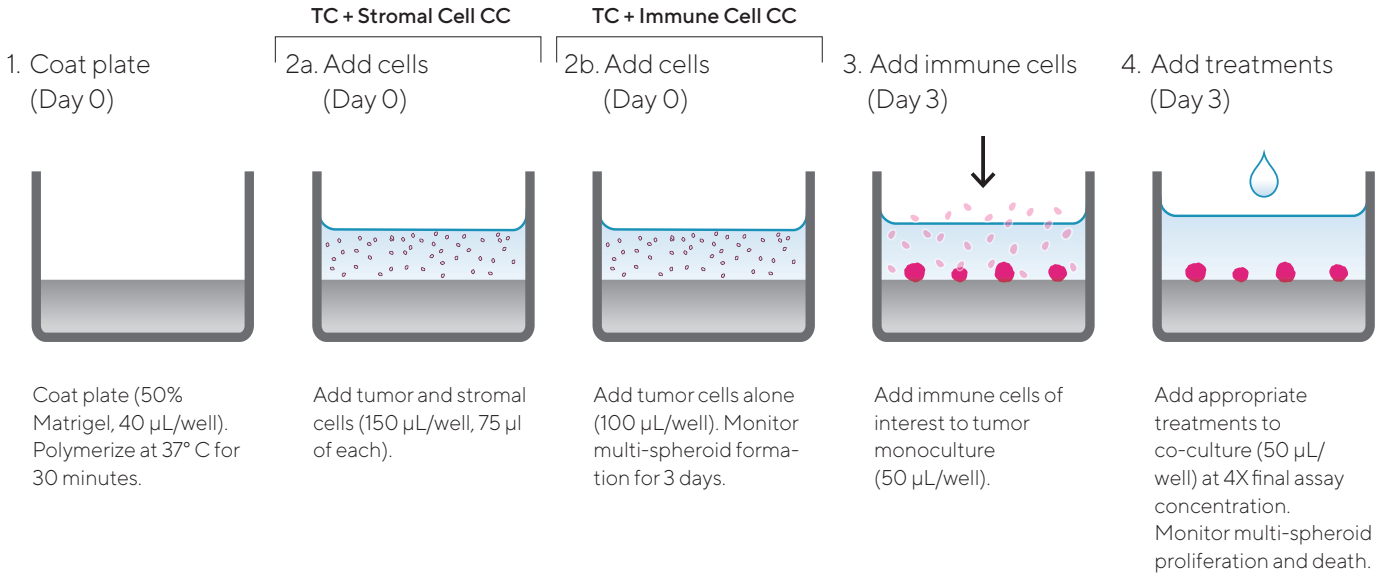

Figure 1: Assay Workflow

1. 96-well micro-titer plate coated with a layer of Matrigel™ (Corning) (40 µL/well, diluted in serum-free media to a minimum concentration of 4.5 mg/mL) and polymerized at 37° C for 30 min
2. Cells of interest harvested, counted and seeded into pre-coated 96-well plates at desired densities (150 or 100 µL/well)
   a. Tumor and stromal cell co-culture: Tumor and stromal cells seeded at 1:1 ratio (150 µL/well, 75 µL of each cell type)
   b. Tumor and immune cell co-culture: Tumor cells seeded alone (100 µL/well)
3. Multi-spheroid formation monitored for 3 d using an Incucyte® Image Analysis system (DF® Brightfield acquisition, 10X magnification, every 6 h)
4. Post multi-spheroid formation, immune cells of interest added to tumor monoculture at an optimized Effector-to-Target ratio (E:T) in a volume of 50 µL/well
5. Appropriate treatments added to co-culture assay plates (50 µL/well) at 4X final assay concentration to achieve a final volume of 200 µL/well
6. Multi-spheroid proliferation was monitored in Incucyte® S3 (6 h repeat scanning) for up to 10 d.

All cell culture reagents were obtained from Life Technologies unless otherwise noted. MDA-MB-231 (ATCC), SK-BR-3 (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). BT-474 (ATCC) cultures were stably transfected with Incucyte® Cytolight Green Lentivirus Reagent (EF1 Alpha Promoter, Puromycin selection, Cat. No. 4481, prepared per Essen BioScience protocol). Lapatinib and Camptothecin were obtained from Sigma, and ZK164015 was obtained from Tocris Bioscience. Normal human dermal fibroblasts (NHDFs) were purchased from CalTag Medsystems (Cat. No. ZHC-5102) and MCF7 Nuclight Red cells from Essen BioScience (Cat. No. 4524). All cell lines were cultured in MEM medium supplemented with 10% FBS, 1% sodium pyruvate plus 1% non-essential amino acids and grown to confluence in 75 cm^2 tissue culture treated flasks (Corning). Cells were harvested and seeded into 96-well plates (Corning No. 3595) coated with a base layer of 40 µL/well Matrigel such that 3 d post cell seeding, multi-spheroids formed with desired size. Spheroid formation was monitored in an Incucyte® S3 over 3 d at 6 h intervals. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy donors using Lymphoprep™ density gradient medium (StemCell Technologies) and maintained in RPMI 1640 supplemented with 10% heat inactivated FBS.

Influence of Stromal Cells on Multi-Spheroid Morphology

NHDFs were shown to have a striking effect on tumor multi-spheroid morphology. SK-BR-3 cells were seeded alone or with NHDFs (1:1 ratio, 1,000 cells/well for each) and DF Brightfield images acquired every 6 h. In mono-culture, SK-BR-3 cells failed to form compact multi-spheroids; co-cultures with NHDFs resulted in the formation of more compact aggregates (Figure 2). The Incucyte® Spheroid Software Module permitted kinetic quantification of both morphologies, tracking multi-spheroid growth via size measurements (Total BF Area). In addition, different temporal growth profiles were revealed through live-cell analysis for a panel of four breast tumor multi-spheroids (MCF7, MDA-MB-231, SK-BR-3, and BT-474) co-cultured with NHDFs (Figure 2 time-course plot).

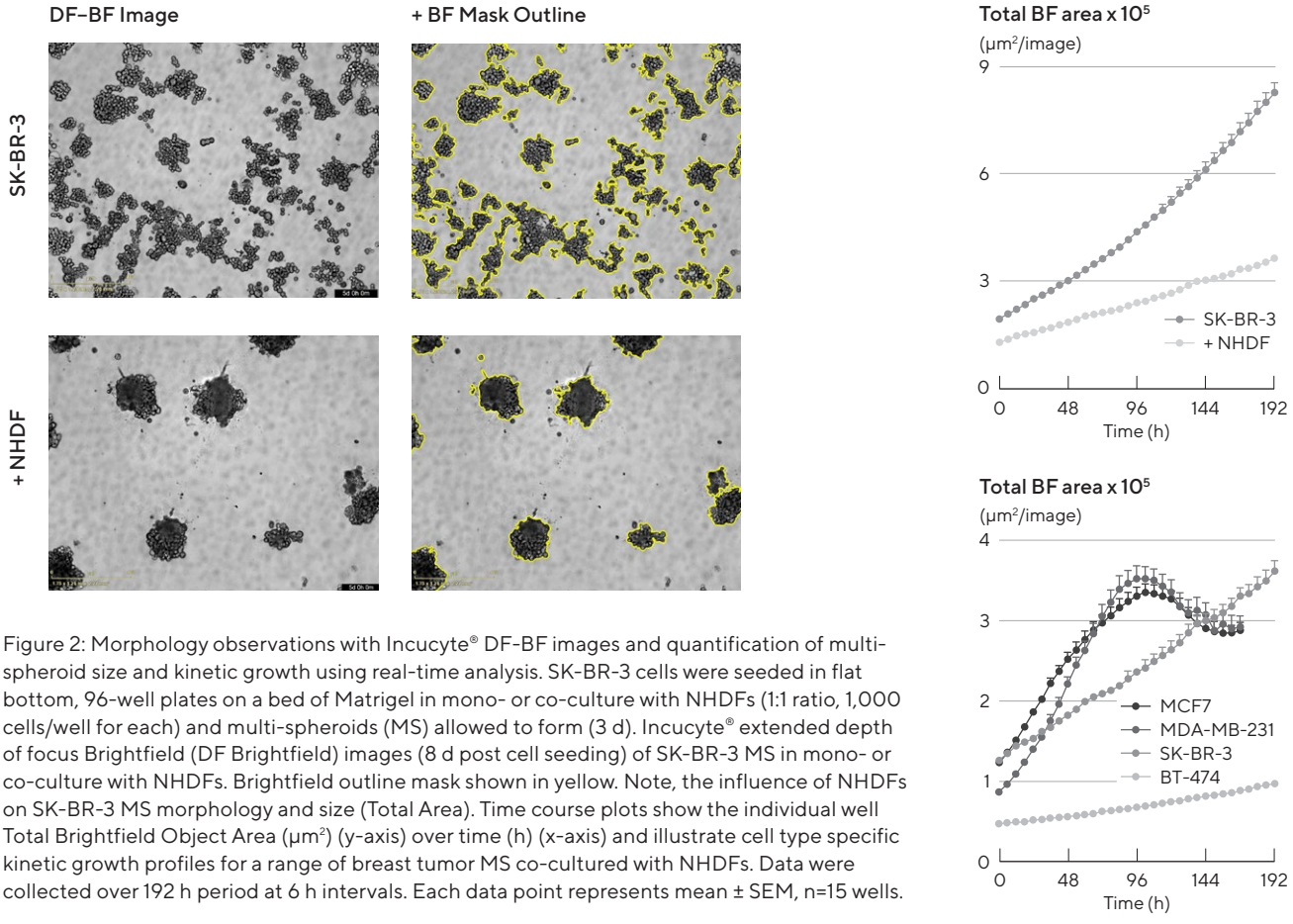

Figure 2: Morphology observations with Incucyte® DF-BF images and quantification of multi-spheroid size and kinetic growth using real-time analysis. SK-BR-3 cells were seeded in flat bottom, 96-well plates on a bed of Matrigel in mono- or co-culture with NHDFs (1:1 ratio, 1,000 cells/well for each) and multi-spheroids (MS) allowed to form (3 d). Incucyte® extended depth of focus Brightfield (DF Brightfield) images (8 d post cell seeding) of SK-BR-3 MS in mono- or co-culture with NHDFs. Brightfield outline mask shown in yellow. Note, the influence of NHDFs on SK-BR-3 MS morphology and size (Total Area). Time course plots show the individual well Total Brightfield Object Area (µm²) (y-axis) over time (h) (x-axis) and illustrate cell type specific kinetic growth profiles for a range of breast tumor MS co-cultured with NHDFs. Data were collected over 192 h period at 6 h intervals. Each data point represents mean ± SEM, n=15 wells.

Interestingly, NHDFs co-cultured with MDA-MB-231 cells (1:1 ratio, 1,000 cells/well for each), revealed a time-dependent effect on spheroid morphology. MDA-MB-231 multi-spheroids transitioned from a stellate, branched appearance, evident at early time-points, to a more clustered round appearance after 6 d (Figure 3).

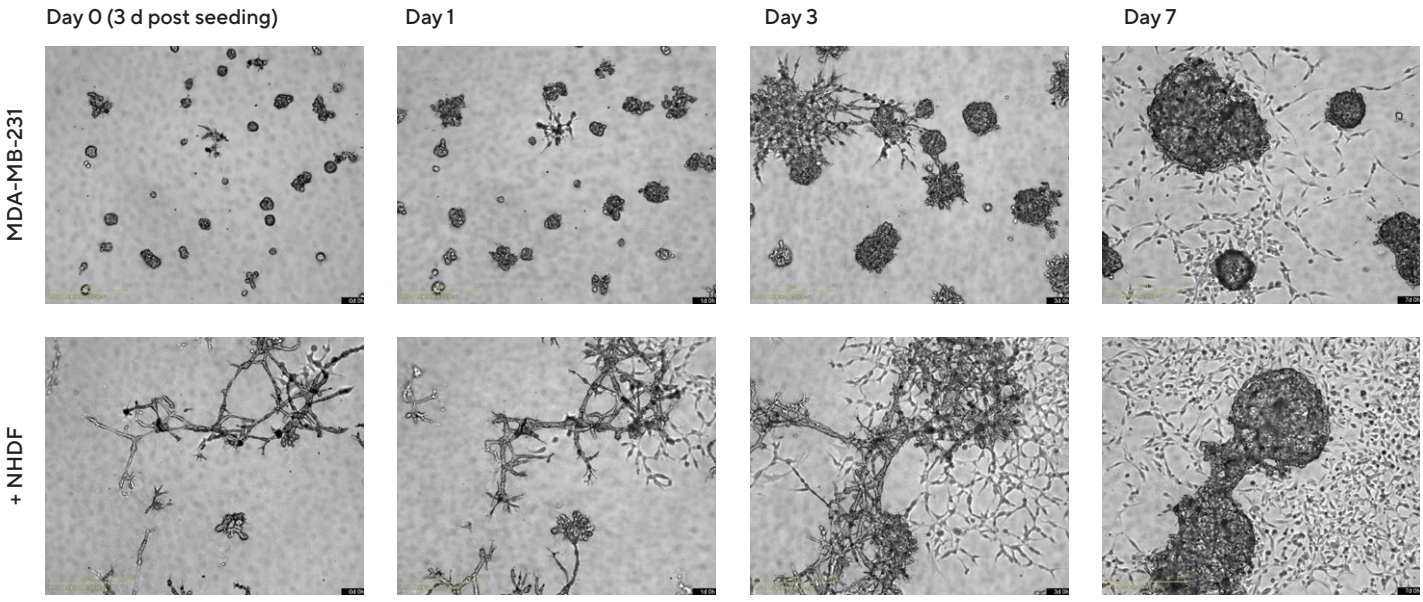

Figure 3: Temporal effects on morphology revealed through Incucyte® DF Brightfield imaging. MDA-MB-231 cells were seeded in flat bottom 96-well plates on a bed of Matrigel in mono- or co-culture with NHDFs (1:1 ratio, 1,000 cells/well for each) and multi-spheroids allowed to form (3 d). Incucyte® S3 DF Brightfield images compare mono- and co-culture conditions over 7 d (10 d post cell seeding). Note the temporal impact of NHDFs on spheroid morphology.

96-Well Multi-Spheroid Growth and Shrinkage Assay for Pharmacological Analysis

To demonstrate the amenability of this 3D tumor and stromal cell co-culture to compound toxicity testing, a pharmacological study was performed using a panel of four commonly used tumor breast cell lines; MCF7, MDA-MB-231, SK-BR-3 and BT- 474. Cells were co-cultured with NHDFs (1:1 ratio, 1,000 cells/ well of each) and multi-spheroids allowed to form for 3 d prior to treatment (up to 10 d) with standard-of-care and cytotoxic agents, Lapatinib, ZK164015, and Camptothecin (CMP).

Incucyte®'s real-time, automated vessel views enabled rapid visualization of treatment effects on multi-spheroid size (Total BF Area). 96-well vessel views of MCF7 multi-spheroids with overlay analysis mask and treatment time-courses illustrate a concentration dependent inhibition of ZK164015 and CMP on spheroid size (Figure 4).

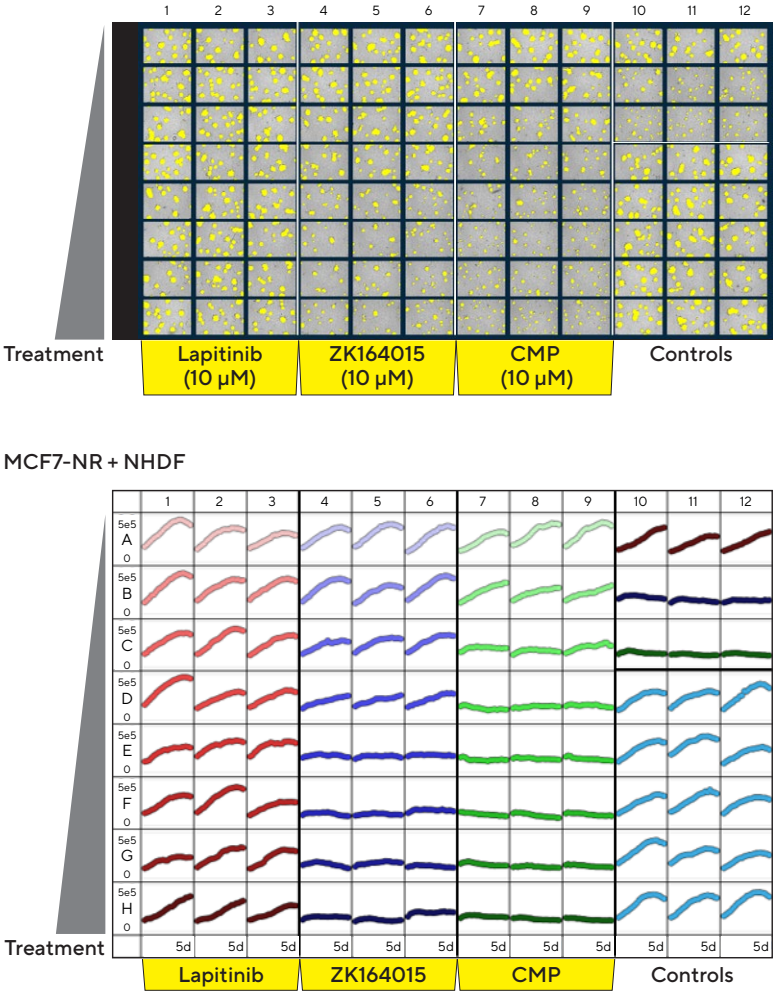

Figure 4: Incucyte® S3 vessel views enable rapid visualization and quantification of treatment effects. MCF7 cells co-cultured with NHDFs were seeded in pre-coated (Matrigel) flat bottom 96-well plates (1:1 ratio, 1,000 cells/well of each) and multi-spheroids allowed to form (3 d). Spheroids were then treated with serial dilutions of known standard of care and cytotoxic compounds (5 d). Incucyte® microplate vessel views show effects of treatments on multi-spheroid size. Top image shows Brightfield Object Area (µm²) segmentation mask (yellow) 5 d post treatment. Bottom image shows the individual well Total Brightfield Object Area (µm²) (y-axis) over time (0–5 d post treatment) (x-axis).

Visualization of brightfield images support specific cytotoxic effects of Lapatinib on SK-BR-3 size and a non-specific cytotoxic effect of CMP on both MCF7 and SK-BR-3 spheroids (Figure 5).

Concentration response curves were constructed using an area under curve (AUC) analysis of the time-course data (Figure 6). Inhibition of multi-spheroid growth caused by Lapatinib and ZK164015 aligned with the known expression profile of receptors targeted by these agents[3]. The dual EGRF and HER2 tyrosine kinase inhibitor, Lapatinib, caused a concentration-dependent inhibition of SK-BR-3 and BT-474 multi-spheroid growth, while the estrogen receptor (ER) antagonist ZK164015 was a potent inhibitor of MCF7 multi-spheroid growth. ZK164015 had little or no effect on multi-spheroids devoid of ER expression. The DNA topoisomerase inhibitor, CMP, caused comparable inhibition of growth across all multi-spheroids types.

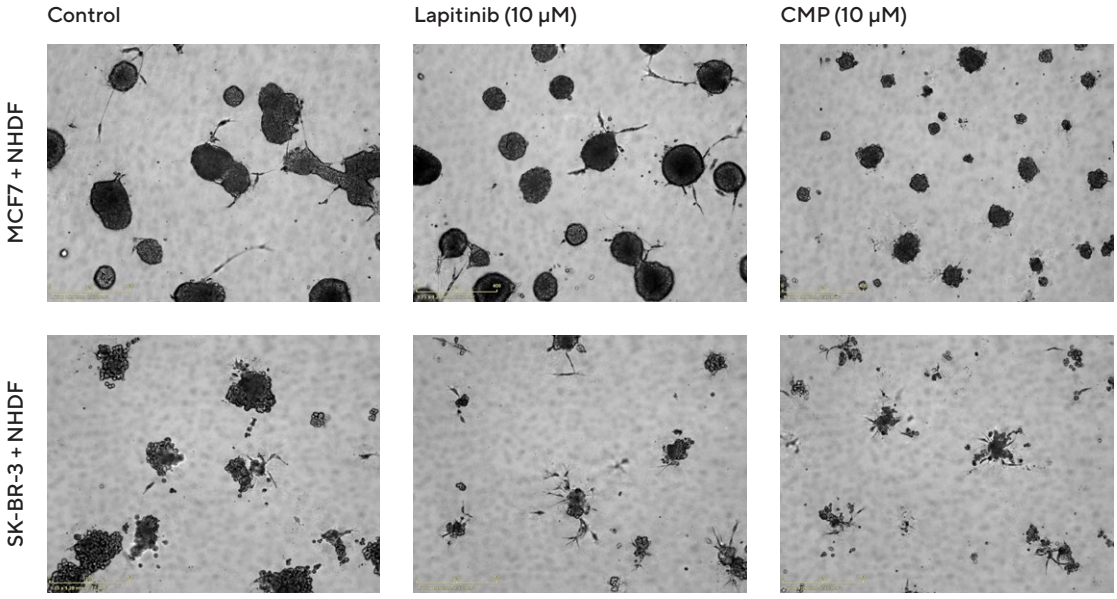

Figure 5: Interrogation of images to gain greater insight. MCF7 or SK-BR-3 cells co-cultured with NHDFs were seeded in pre-coated (Matrigel) flat bottom 96-well plates (1:1 ratio, 1,000 cells/well of each) and multi-spheroids allowed to form (3 d) prior to treatment with Lapatinib and Camptothecin (CMP). Incucyte® Brightfield images (5 d) show the effects of treatments on multi-spheroid size and integrity. Note: cytotoxic effects of CMP on both MCF7 and SK-BR-3 multi-spheroids and their difference in sensitivity to Lapatinib.

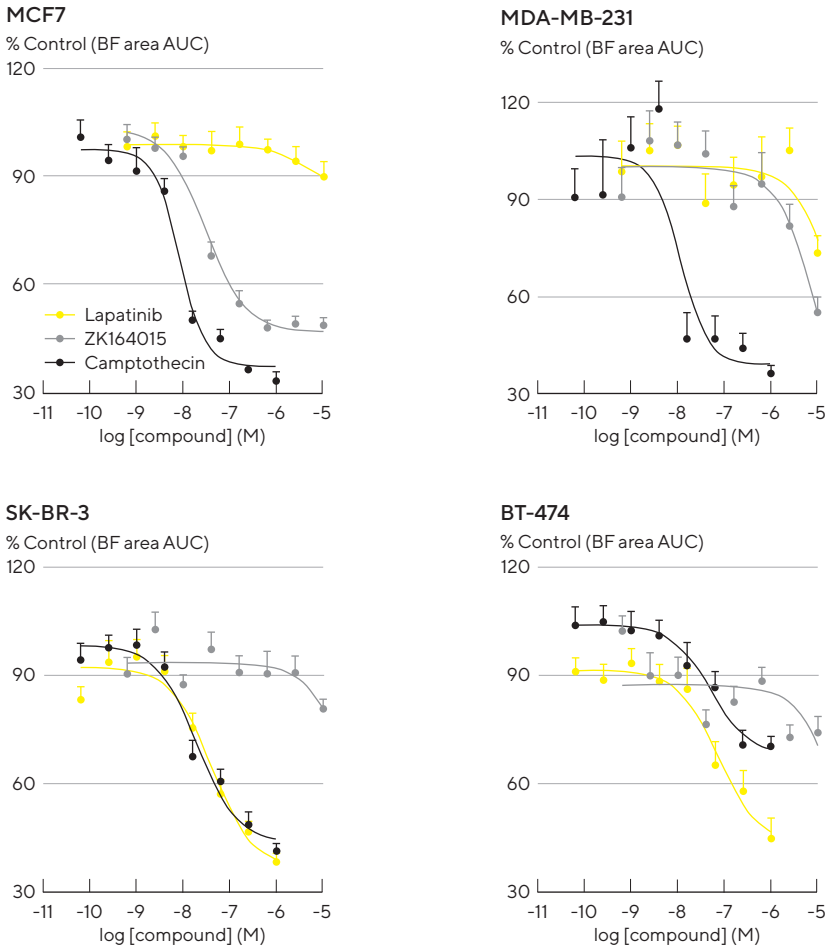

Figure 6: Robust and reproducible pharmacological analysis through generation of concentration response curves. A panel of four tumor breast cell lines were seeded in 96-well flat bottom plates with NHDFs (1:1 ratio, 1,000 cells/well for each on a bed of Matrigel). Multi-spheroids were allowed to form for 3 d prior to treatment with standard-of-care and cytotoxic agents. Concentration response curves (CRCs) represent the area under curve (AUC) of the Total Brightfield Area (μm^2) time course data (not shown) from 0–6 d (MCF7, MDA-MB-231) or 0–10 d (SK-BR-3, BT-474) post-treatment. Each data point represents mean ± SEM, n=9–12 wells. Note: differential compound pharmacology across cell types.

Herceptin®-Induced ADCC in HER2-Positive Multi-Spheroids

The regulation of immune-mediated killing by monoclonal antibodies is an important mechanism of action in immunotherapy treatments[4]. Binding of antibodies such as Herceptin (a humanized monoclonal antibody targeting HER2 receptor) to receptors induces antibody-dependent, cell-mediated cytotoxicity (ADCC).

The impact of Herceptin-induced ADCC was evaluated in HER2-positive (SKOV3 and BT474) and HER2-negative (MCF7) multi-spheroids. Tumor multi-spheroids, either stably expressing nuclear restricted RFP (NR) or cytoplasmic GFP (CyG), were co-cultured with freshly isolated peripheral blood mononuclear cells (PBMCs) (E:T, 5:1) and treated with Herceptin. Multi-spheroid proliferation and immune cell-mediated cytotoxicity were quantified kinetically using fluorescence intensity within brightfield boundary, which does not require masking of fluorescent tumor multi-spheroid.

PBMCs alone (data not shown) or Herceptin alone had little or no effect on multi-spheroid size or viability (fluorescence intensity, Figure 7). However, Herceptin activated PBMCs, resulting in a marked loss of MS viability.

Herceptin induced a concentration-dependent, cytotoxic effect in HER2-positive multi-spheroids but not HER2-negative multi-spheroids. BT-474 multi-spheroids appeared more sensitive to Herceptin cytotoxicity, with the highest test concentration of Herceptin (1 µg/mL) producing approximately 80% inhibition compared with approximately 50% in SKOV3 multi-spheroids (Figure 8, concentration response curve). Activation of the PBMCs T cell population with an AntiCD3 antibody and IL-2 cocktail (10 ng/mL of each) resulted in maximal killing across all cell types (Figure 8, time-courses).

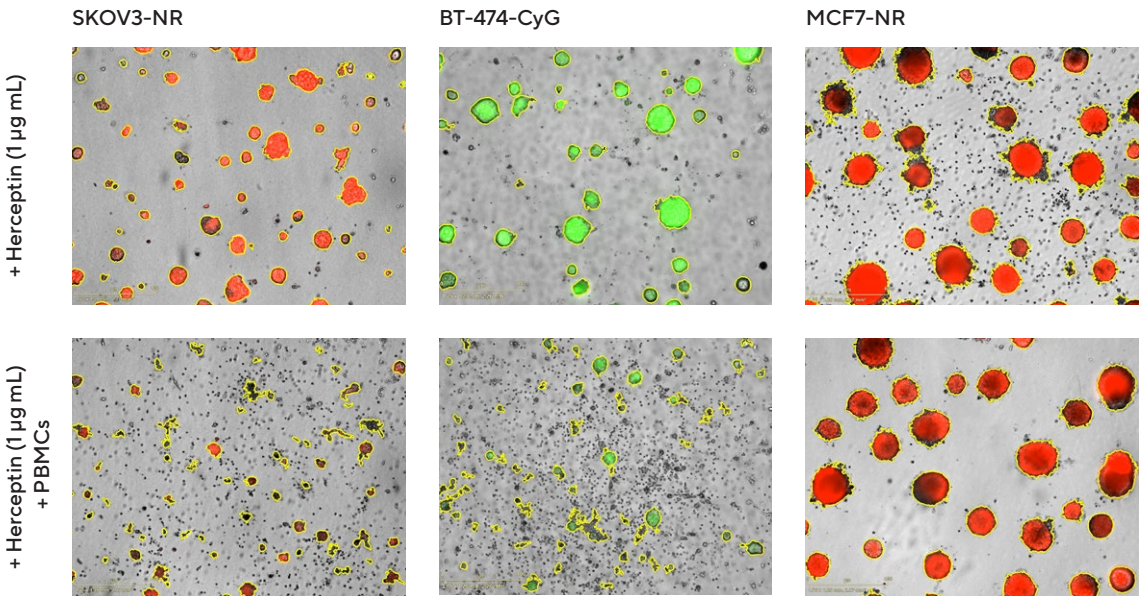

Figure 7: Impact of Herceptin-induced PBMCs on multi-spheroid proliferation. Tumor cells either stably expressing nuclear restricted RFP (SKOV3-NR, MCF7-NR) or cytoplasmically restricted GFP (BT-474-CyG) were seeded in flat bottom 96-well plates (1,000 cells/well on a bed of Matrigel). Multi-spheroids were allowed to form (3 d) prior to addition of freshly isolated PBMCs (E:T, 5:1) and Herceptin. Incucyte® S3 brightfield and fluorescence images (7 d; SKOV3-NR, MCF-NR or 10 d; BT-474-CyG) compare the effect of Herceptin on spheroid proliferation in absence (top panel) and presence (bottom panel) of PBMCs (Brightfield outline mask shown in yellow). Note the loss of fluorescence intensity in HER2-postive (SKOV3 and BT474) multi-spheroids in the presence of PBMCs.

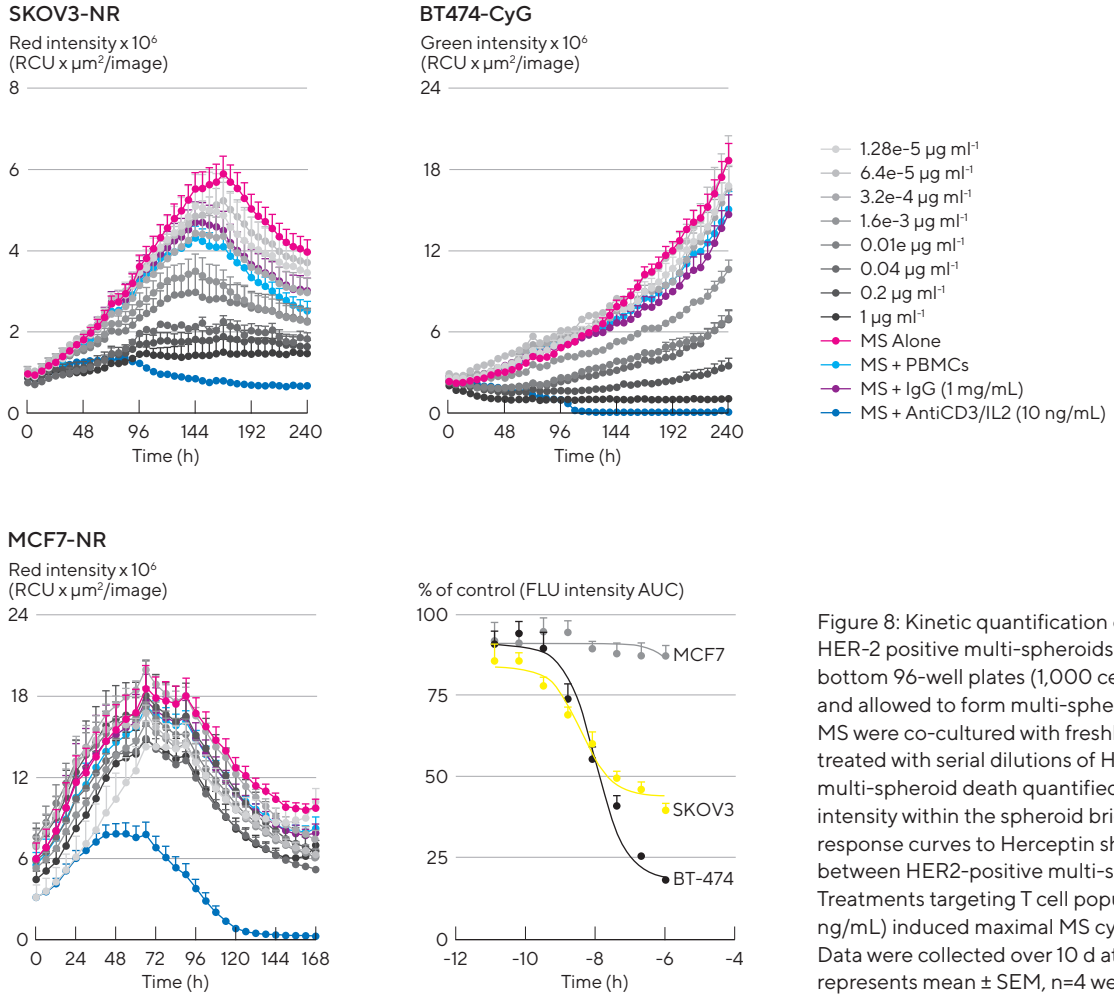

Figure 8: Kinetic quantification of ADCC immune cell killing of HER-2 positive multi-spheroids. Tumor cells were seeded in flat bottom 96-well plates (1,000 cells/well on a bed of Matrigel) and allowed to form multi-spheroids (MS) for 3 d. Once formed, MS were co-cultured with freshly isolated PBMCs (E:T, 5:1) and treated with serial dilutions of Herceptin. Time-courses show multi-spheroid death quantified as a loss of fluorescence intensity within the spheroid brightfield object. Concentration response curves to Herceptin show sensitivity differences between HER2-positive multi-spheroids (SKOV3 and BT-474). Treatments targeting T cell populations (Anti-CD3 and IL-2, 10 ng/mL) induced maximal MS cytotoxicity across all cell types. Data were collected over 10 d at 6 h intervals. Each data point represents mean ± SEM, n=4 wells.

Conclusions

In this application note, we demonstrate that the Incucyte® Live-Cell Analysis System, in conjunction with the Incucyte® Spheroid Software Module, enables the analysis of 3D multi-spheroid co-cultures with either stromal or immune cells over time and is applicable to compound testing. We have shown:

- Live-cell analysis demonstrated that SK-BR-3 formed more compact multi-spheroids when cultured with NHDFs
- Live-cell imaging revealed temporal effects of NHDFs on MDA-MB-231 multi-spheroid morphology
- Cell type specific temporal growth profiles were elucidated for a panel of breast tumor multi-spheroids
- The capability to perform real-time compound profiling in a 96-well format
- The ability to kinetically visualize and quantify antibody-dependent cell-mediated cytotoxicity (ADCC) immune cell-induced within target tumor multi-spheroids in real-time
- Herceptin activated PBMCs caused a concentration dependent loss of HER2 positive multi-spheroid viability

More advanced 3D models, incorporating an extracellular matrix and additional cell types (e.g., stromal cells or immune cells) have the potential to provide more relevant translational models for the study of the tumor microenvironment on tumor biology.

Several features of the Incucyte® Live-Cell Analysis System are particularly advantageous for monitoring and objectively quantifying 3D multi-spheroid biology in real time. The DF Brightfield imaging allows for label-free study of 3D spheroid morphology and proliferation in 96-well assay formats for enhanced throughput. With no need for selection of a predefined endpoint, consistent segmentation and quantification of brightfield images enables kinetic assessment of cell-dependent growth profiles, and the impact of stromal cells on tumor multi-spheroid resistance to chemotherapeutic agents. Brightfield in combination with fluorescence imaging permits visualization and quantification of immune cell-mediated toxicity of tumor multi-spheroids.

Incucyte®'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 provide a unique and efficient technical platform that can be incorporated into existing workflows.

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