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Innovations in Live-Cell Imaging: Harnessing Confocal Technology for Enhanced 3D Biological Insights

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Abstract

Three-dimensional (3D) complex cell models such as spheroids, organoids, and co-culture systems are increasingly central to oncology and immunology drug discovery because they capture structural complexity and translational behavior that two-dimensional (2D) monolayer models cannot. However, widefield imaging and endpoint assays fall short in quantifying 3D biology due to a lack of optical sectioning, depth resolution, and quantitative fidelity that obscures biological data. These limitations introduce uncertainty into drug development decisions.

This whitepaper will investigate the advantages of confocal live-cell imaging and introduce the Incucyte® CX3 Live-Cell Analysis System (Incucyte® CX3). This system aims to overcome the inherent limitations of widefield and endpoint methods by providing depth-resolved visualization of complex cell models, enabling more accurate quantification, and more reliable biological insights at throughput. Incucyte® CX3 resolves spatial and kinetic biology directly within the incubator, enabling continuous, long-term, Z-resolved imaging of 3D models as they develop and respond to treatment. The system combines spinning-disk confocal fluorescence with an intuitive user experience, guiding users from acquisition setup to data visualization, ensuring every experiment produces meaningful, reproducible results. By monitoring complex 3D biology in real time, researchers gain earlier insight into drug effects and biological mechanisms, leading to stronger go and no-go decisions and more efficient discovery workflows.

Introduction

The Shift Toward Physiologically Relevant 3D Models

Drug discovery has steadily shifted toward more physiologically relevant 3D cell models, as their spatial organization and heterogeneity better mimic *in vivo* conditions. These models develop structures, gradients, and reach states like those found *in vivo* over several days, which are difficult to capture at single time points or accurately measure with standard widefield microscopy.[1,2] Their responses to treatments also unfold gradually, affecting their shape, viability, and functional behavior, necessitating ongoing monitoring. This prolonged observation is especially critical in fields like immuno-oncology, antibody therapeutics, and regenerative medicine, where complex interactions, delayed mechanism of action, and maturation-dependent behaviors highlight the need for imaging approaches that can resolve 3D biological development and responses over time.

As drug discovery progresses towards more intricate multicellular systems, imaging tools must adapt to meet these new demands. Researchers need technologies that can accurately resolve complex 3D biology while maintaining the necessary conditions for long-term, live-cell studies. Confocal imaging within incubators offers a solution, addressing these requirements effectively.[3,4]

Confocal Imaging in a Live-Cell Environment

Principles of Live-Cell Confocal Imaging

Live-cell confocal imaging improves visualization of thick biological samples by using optical sectioning to capture fluorescence from a narrow focal plane while rejecting out-of-focus light.[5] This selective detection increases axial resolution and preserves structural detail across the Z-axis, which is essential for accurately interpreting cellular organization in 3D tissues.

Spinning-disk confocal systems employ an array of rapidly rotating pinholes that sample many points in parallel. This design minimizes photobleaching and phototoxicity compared to point-scanning systems, allowing sensitive cell models such as spheroids, organoids, and immune co-cultures to be imaged repeatedly over extended periods without perturbing their physiology. The ability to acquire Z-stacks supports reconstruction of complex 3D architectures and detection of depth-dependent biological events.

By delivering consistent optical sectioning, and higher contrast, live-cell confocal imaging, this strengthens segmentation and quantification of cell health and function within denser 3D biological models. This results in more accurate measurement of proliferation, apoptosis, immune cell interactions, and other dynamic processes that might be obscured or inaccurately represented with widefield imaging.

Incucyte® CX3 Live-Cell Analysis System Overview

The Incucyte® CX3 is designed to provide advanced biological insights through straightforward and automated workflows. With its incubator-integrated design, it enables continuous, non-invasive imaging that allows researchers to monitor dynamic cellular behaviors without disturbing the culture conditions. This feature is especially beneficial for studying complex biological systems where understanding kinetic and spatial information is crucial for accurate interpretation.

- **Incubator-integrated environment** that maintains physiological temperature, humidity, and CO_2, and supports long-term observation of both 2D monolayers and 3D spheroids, organoids, and immune co-culture systems without perturbation.
- **Enhanced optical performance** provides the clarity and contrast required to resolve subtle phenotypic changes, cell state transitions, and treatment-induced effects in both 2D and 3D models.
- **Multi-channel fluorescence imaging (green/orange/near-infrared)** supports simultaneous readouts of viability, apoptosis, immune engagement, cell cycle progression, and other mechanistic markers relevant to oncology, immunology, and cell therapy research.
- **Automated Z-stack acquisition** enables depth-resolved imaging of heterogeneous 3D structures and supports

applications such as monitoring tumor spheroid growth and invasion, studying immune cell interactions, assessing responses to treatments, and observing morphological changes.

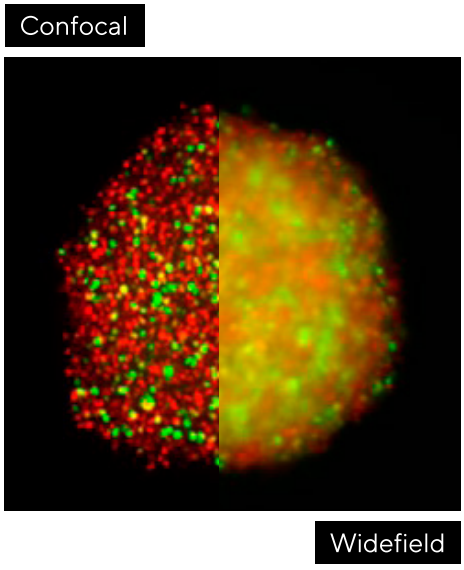
- **Higher-throughput walk-away workflows** streamline the execution of experiments and support screening-scale studies in 96- and 384-well formats, with a 6-plate throughput. This setup enables users to produce complex, spatially resolved datasets with minimal hands-on involvement, effectively handling large-scale experiments.

- **Integrated kinetic analysis software** provides object-level quantification and temporal profiling and helps users characterize proliferation dynamics, killing kinetics, morphological changes, and pathway responses with statistical confidence.

By delivering confocal-quality data through an intuitive and automated platform, the Incucyte® CX3 allows researchers to uncover deeper biological insights, including mechanism of action, temporal response patterns, and spatial cell behaviors without requiring specialized imaging expertise. This combination of scientific rigor and operational simplicity positions the system as a powerful tool for accelerating discovery in oncology, immuno-oncology, and advanced cell therapy research.

Confocal vs. Widefield Performance in 3D Models

Confocal imaging offers significant benefits for 3D cell biology by isolating light from a single optical plane and eliminating out-of-focus signals.[5,6] Key features, listed in Table 1, combine to improve the clarity of imaging 3D cell models. By providing higher contrast and improved signal clarity via optical sectioning, confocal imaging enables more precise quantification of cell populations, phenotypic markers, and dynamic processes. This enhanced optical clarity supports reliable measurement of markers of cell health, cell interactions, and subtle phenotypic and treatment responses in complex 3D models, delivering deeper biological insight than traditional widefield or endpoint fluorescence imaging.



Feature	Widefield Fluorescence	Spinning Disk Confocal
Illumination	Broad, uniform light excites the entire sample	Focused laser scans a single optical plane at a time
Signal Detection	Collects all emitted light-both in and out-of-focus	Detects only in-focus light through a pinhole (spinning disk)
Resolution	Suitable for monolayers and thin samples	Superior axial resolution ideal for thick 3D models
Depth penetration of 3D samples	Detects fluorescence across depth, but no reliable per-layer resolution	50-100 µm (~5 cell layers deep, depending on laser and cell size)
Recommended use	2D culture, adherent cells, migration assays	3D organoids, spheroids, co-cultures and dense tissues

Table 1. Confocal Imaging Delivers Superior clarity and Depth Resolution in 3D models

Applications in Drug Discovery Workflows Using Incucyte® CX3 Live-Cell Analysis System

The Incucyte® CX3 enables high-fidelity visualization and quantification of complex cellular behaviors in 3D systems. By integrating optical sectioning with continuous live-cell monitoring, the system supports mechanistic insight across diverse biological workflows, including cell cycle regulation, immune–tumor interactions, cytotoxicity, and organoid development.

Accurate 3D Cell Cycle Analysis

Quantifying cell cycle progression within 3D tissues is challenging because densely packed nuclei and depth-dependent signal mixing in widefield images can obscure accurate boundary detection. The confocal Z-stack acquisition supported by the Incucyte® CX3 improves axial resolution, enabling more precise segmentation of individual nuclei and more reliable classification of fluorescent reporters that mark distinct cell cycle phases.
To illustrate how imaging modality differences impact biological interpretations, U87 MG spheroids stably expressing Incucyte® Cell Cycle Green/Orange construct were generated over three days and subsequently exposed to 5-fluorouracil (5-FU) to induce G1 arrest. Replicate plates were imaged over five days using either the Incucyte® SX5 widefield system or the CX3 spinning-disk confocal system.

The confocal maximum-intensity projection images demonstrate the expected biological response (Figure 1). Treatment resulted in an increased orange signal indicating G1 arrest, accompanied by a reduction in the green fluorescence associated with S/G2/M phases.
In contrast, the widefield single-plane images appeared to show a progressive increase in green fluorescence over time, which is an artifact arising from spectral unmixing limitations, the out-of-focus light, and elevated background inherent to the widefield imaging. These features explain why multi-fluorescent imaging is not recommended with Incucytes' current widefield imaging. This practical example highlights how confocal imaging mitigates these artifacts, providing a more accurate representation of cell-cycle dynamics in 3D spheroid models.

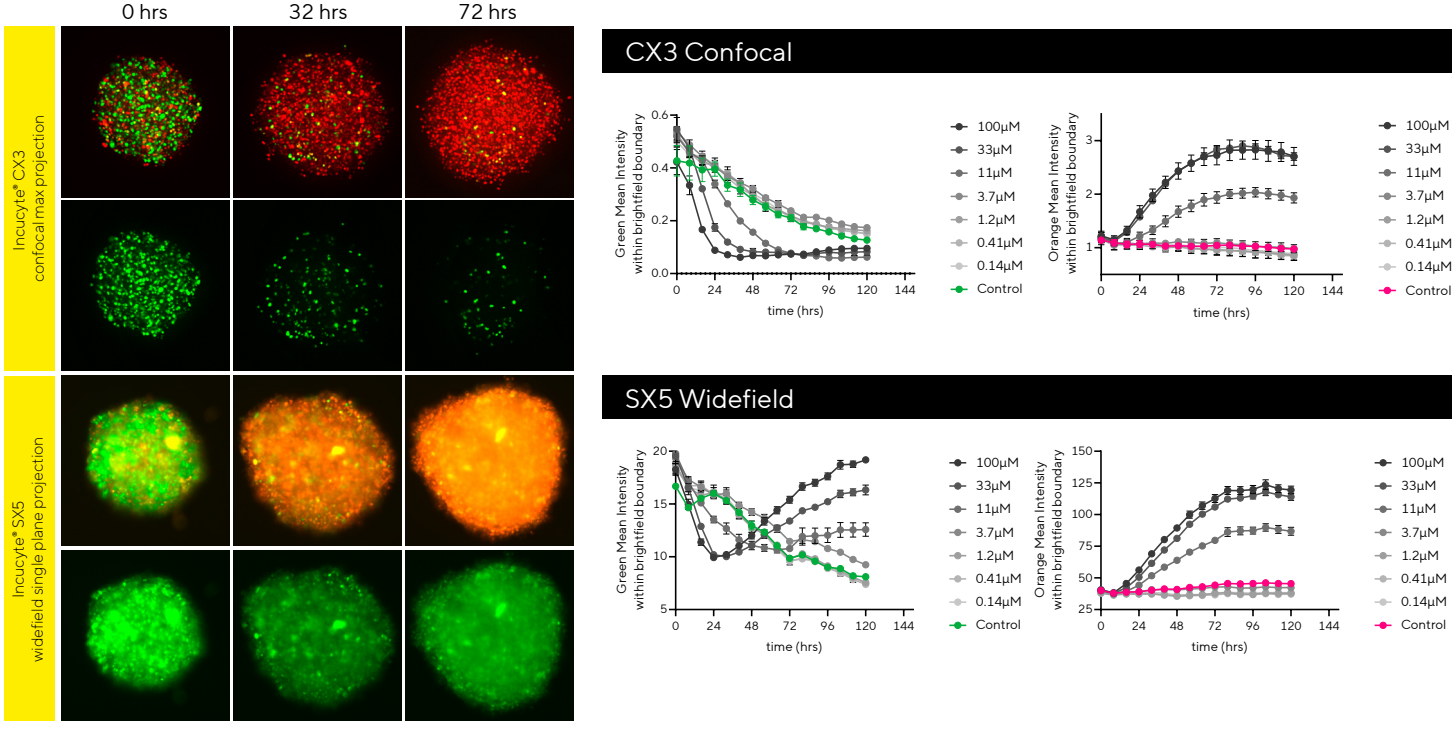


Figure 1. Confocal Imaging Delivers Clearer, More Reliable 3D Cell Cycle Readouts. Green and Orange Channel and Green channel alone fluorescence images of U87 MG spheroids stably expressing the Incucyte® Cell Cycle Green/Orange reagent post 3-day generation collected on either Incucyte® CX3 (max projection confocal fluorescence image) or SX5 (widefield fluorescence image) Systems with associated quantification of orange (G1) and green (S/G2/M) signal over time in response to 5-FU. Data shown as mean ± SEM of 4 wells.

Immune Cell Killing in 3D (T-cell and NK-cell Assays)

Immune-mediated tumor killing, whether by T-cells or through antibody-dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells within 3D tumor spheroids, require accurate visualization of effector–target interactions.[2] This includes observing immune-cell infiltration and localized tumor-cell killing. Widefield imaging often fails to resolve these events due to out-of-focus blur which causes immune cells and tumor tissue to appear merged, particularly within deeper spheroid layers. This can obscure the dynamics of effector engagement and hinder reliable masking of early cytotoxic responses.

The Incucyte® CX3, through confocal imaging, enhances resolution at the spheroid interface and within the outer cell layers, facilitating spatial differentiation of immune cell accumulation and early infiltration events that widefield fluorescence imaging cannot reliably distinguish. This improved spatial information aids in interpreting immune-mediated effects in 3D tumor models and detecting the onset of cytotoxic activity in specific regions. By resolving spatial and temporal heterogeneity in treatment responses, the CX3 supports the creation of detailed kinetic profiles, which more accurately reflect antibody potency, immune mechanisms of action, and the complex biology inherent in 3D tumor microenvironments.

To illustrate this, a single spheroid immune killing model was assessed using tumor spheroids generated using HER2 positive SKOV3 cells stably expressing a nuclear restricted fluorescent orange protein (Incucyte® Nuclight Orange 2.0). Spheroids were generated over three days, after which NK cells were introduced using a 2.5:1 effector-to-target (E:T) ratio in the presence of trastuzumab or IgG1 control (10 µg/mL).

Immune cells were labeled with Incucyte® Fabfluor-488–conjugated CD45 in combination with a background suppressor, Incucyte® Opti-Green, and Incucyte® Annexin V NIR to indicate cell apoptosis.

Imaging was conducted every six hours using confocal multiplane acquisition at 10× magnification capturing fluorescence across three channels (Figure 2). Under these conditions, the contrast between imaging modalities becomes especially pronounced. As mentioned previously, widefield imaging for multiple fluorescence channels is not recommended. Multiplexed signals become difficult to differentiate, as spectral overlap between the orange and green channels results in significant loss of the green signal, which prevents reliable quantification of individual populations. As a result, for widefield data it is recommended to only use fluorescent measurements in one channel for example only using fluorescence from the spheroid.
In contrast, spinning-disk confocal imaging yields a markedly improved outcome. By using focused laser-based excitation to reduce the need for spectral unmixing of signals in combination with optical sectioning, out-of-focus light is excluded, resulting in substantially higher signal-to-noise ratios and enabling reliable fluorescence multiplexing in complex 3D samples. This enables clear discrimination of labeled immune cells from the tumor spheroid and allows their spatial and temporal interactions to be quantified.
Using confocal imaging, the Incucyte® CX3 resolves T-cell and NK-cell distribution at and near the spheroid surface, enabling measurement of immune cell accumulation in outer spheroid layers and time-resolved assessment of tumor cell apoptosis. This added spatial context improves interpretation of immune-mediated effects and supports differentiation of immunotherapy mechanisms of action

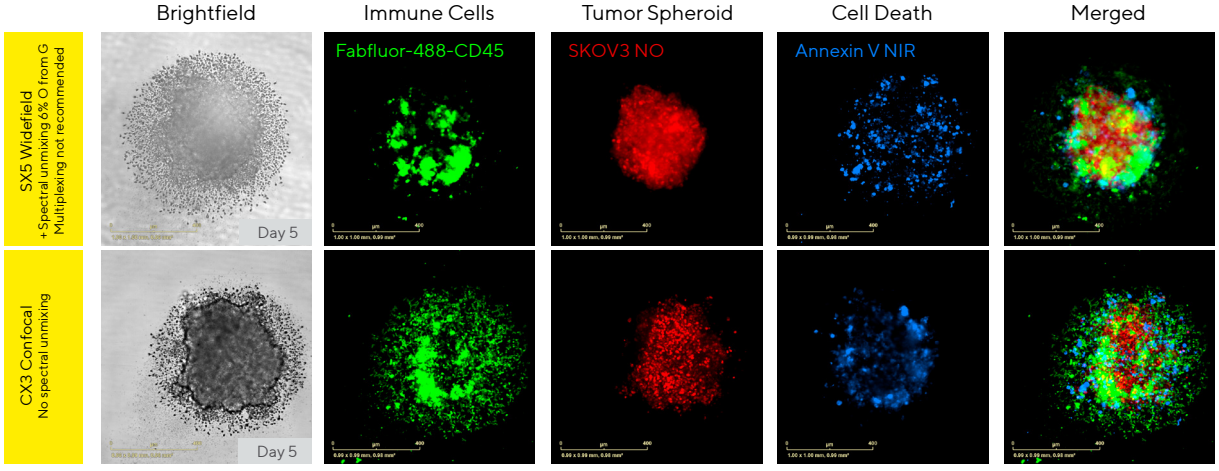


Figure 2. Improved Signal-to-Nosie and Multiplexing in 3D Immune Cell Killing Spheroid Assays. SKOV3 Nuclight Orange tumor spheroids were co-cultured with NK cells (2.5:1 E:T) treated with trastuzumab or IgG control in the presence of Fabfluor-488-CD45, Opti-Green background suppressor, and Annexin V NIR. Representative 10x brightfield and fluorescent images are shown for SX5 widefield (multiplexing not recommended) or CX3 confocal acquisition at 5 days post-treatment.

Multi-Spheroid Assays

Multi-spheroid cultures support population-level readouts, revealing variability in growth, drug response, and invasive behavior. However, the value of these 3D assays relies heavily on the quality of the imaging modality used to extract quantitative endpoints.[7] Widefield imaging presents known challenges under these conditions, such as out-of-focus blur which causes adjacent spheroids to appear fused and structural features at different depths to be projected into a single plane. Different assay protocols can be utilized to minimize multi-plane spheroids such as "on-top" but users still risk segmentation algorithms misidentifying boundaries, underestimate spheroid area or volume, or even merge multiple structures into a single object which directly reduces the accuracy of measuring biologically significant heterogeneity. Confocal imaging addresses these limitations by generating depth-resolved image stacks that preserve the true spatial positioning of each spheroid within a dense population. By rejecting out-of-focus light, it is possible to produce a clean maximum-intensity or sum projection image that maintains clear separation between microtissues and retains fine morphological detail across the Z-stack.

To illustrate this, multi-spheroids formed from BT474 cells stably expressing Incucyte® Cytolight Green were treated with, an IgG control, trastuzumab, and the anti-HER2 antibody-drug conjugates (ADCs) Kadcyla® and Enhertu across a range of concentrations, together with Incucyte® Annexin V NIR. Green and NIR fluorescent confocal images were then collected using the Incucyte® CX3. Both Kadcyla and Enhertu reduced green fluorescence intensity from the spheroid in this model indicating cell death and produced a concentration-dependent increase in Annexin V binding (Figure 3). In comparison, trastuzumab, the monoclonal antibody on which the two ADCs are based, induced a very large reduction in green and increase in NIR intensity at the highest concentration tested, with a more moderate response compared to the ADCs across the rest of the concentration range.

In drug screening applications, reliable detection of small, fast-growing, or irregularly shaped spheroids becomes increasingly important. With improved imaging and quantification misleading results can be reduced and metric stability across replicates improved. Depth-resolved imaging also enhances the accuracy of derived measurements, such as volume, circularity, invasion distance, and fluorescence intensity, thus allowing subtle phenotype differences to be captured with greater confidence. Together, these capabilities position confocal live-cell imaging as a highly effective solution for multi-spheroid assays, supporting higher-throughput workflows without compromising spatial fidelity or biological interpretability.

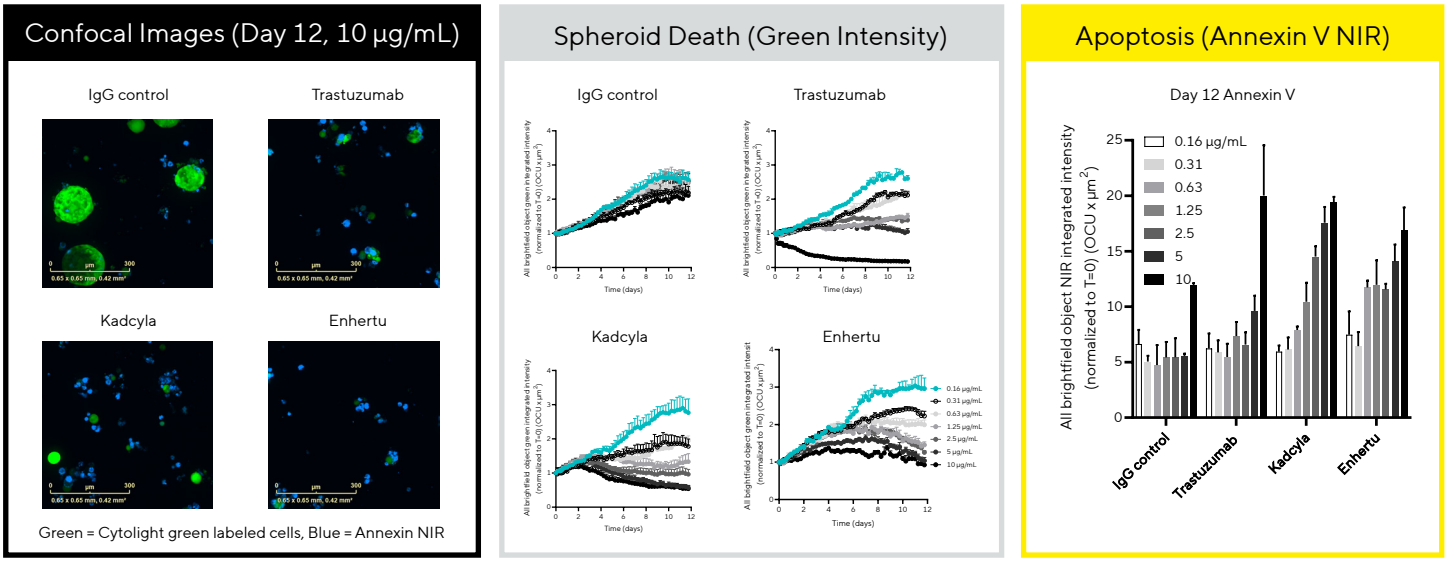


Figure 3. Antibody-Drug Conjugate (ADC) Induced Killing in a Multi-Spheroid Model. BT474 cells expressing Cytolight Green were seeded on top of a layer of Matrigel® for 7 days to promote multi-spheroid formation. Either IgG control, trastuzumab, Kadcyla or Enhertu were added to spheroids at a range of concentrations alongside an Incucyte® Annexin V Dye (NIR). Confocal images with green and NIR fluorescence were captured every 6 hours until day 12. (left panel) Images of multi-spheroids taken on day 12 showing the effects of 10 µg/mL of each antibody. (middle panel) Concentration response curves showing time course data for spheroid green integrated intensity (normalized to the first time point) in the presence of each antibody. (right panel) Annexin NIR intensity on day 12 (normalized to the first time point). All data shown as a mean of 3 wells + SEM.

Organoid Assays

Organoids possess intricate architectures, often featuring lumens, branching networks, and folded epithelial layers, which are challenging to image accurately with widefield modalities.[5] Widefield systems lack optical sectioning, leading to signals from cells across multiple planes blurring together, obscuring subtle changes over time.[8] This limitation hinders accurate tracking of cell behavior or treatment responses in live, thick samples. Confocal imaging addresses these challenges by providing depth-resolved, plane-specific imaging that captures growth, differentiation trajectories, and morphological transitions over time. Beyond the existing Incucyte label-free widefield quantification of organoids, the CX3 supports confocal imaging for visual clarity and enables the capture and quantification of fluorescence signals.

To demonstrate the advantages of confocal live-cell analysis in monitoring organoid biology over time, murine hepatic organoids expressing Incucyte® Nuclight Orange were embedded in a matrix and cultured for one day before exposure to varying concentrations of camptothecin (CMP) or cisplatin (CIS) to induce cell death. Fluorescent images acquired using the Incucyte® CX3, in the presence of Annexin V Green Dye, captured the organoids' morphology immediately before and three days post-treatment, revealing distinct structural changes and corresponding increases in cell death (Figure 4).

The CX3's capability to measure increases in mean green fluorescence intensity in both CMP- and CIS-treated organoids indicates a concentration-dependent decrease in viability and an increase in cell death, corroborated by concentration response curves.

This demonstrates how CX3 confocal imaging can uncover genuine biological responses over time using both label-free morphology readouts and two-color fluorescence. This enhances mechanistic studies across precision oncology, developmental biology, and regenerative medicine by providing the spatial fidelity and temporal continuity needed to track complex organoid phenotypes within a scalable, assay-ready workflow.

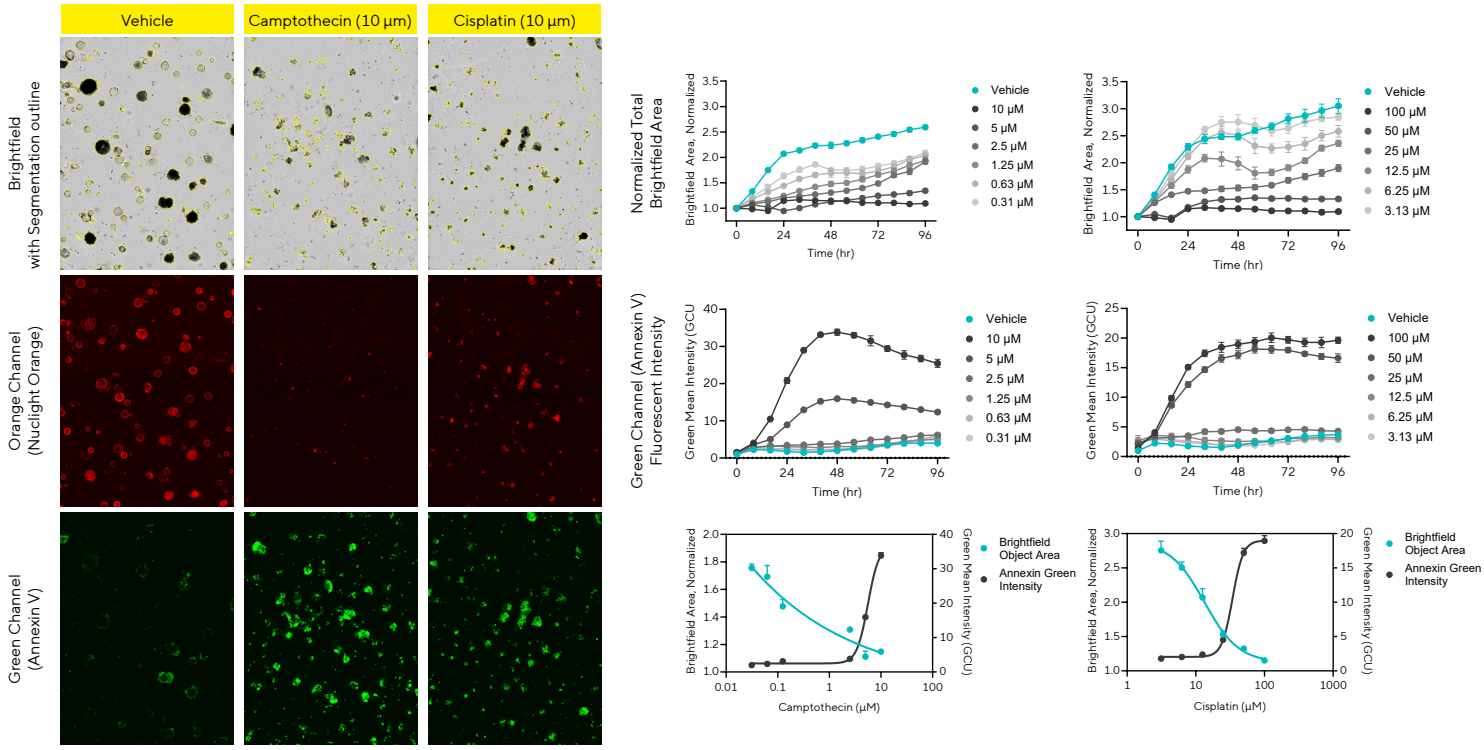


Figure 4. Continuous, Multiplexed Analysis of Hepatic Organoid Drug Response. Nuclight Orange-expressing murine hepatic organoids were embedded in a matrix and cultured for one day prior to treatment with varying concentrations of Camptothecin (CMP) or Cisplatin (CIS). The organoids were subsequently imaged over an additional three days in the presence of Annexin V Green. Images are shown in Brightfield (top), Orange (middle), and Green (bottom) channels, displaying the effects of the indicated compounds at three days post-treatment. Graphs illustrate an increase in Mean Green Fluorescence Intensity in organoids treated with CMP and CIS. The EC^{50} values for CMP and CIS, assessed one day post-treatment, reveal a concentration-dependent decrease in viability and an increase in cell death

Object-Based Analysis of 3D models

The Incucyte® CX3, together with the 3D Object Classification Software Module, enables automated identification and classification of individual objects in heterogeneous live-cell 3D cultures. Leveraging confocal imaging, each spheroid or organoid within a heterogenous cultures can be segmented, allowing precise quantification of size, shape, and fluorescence characteristics.

This fluorescent multiplexed resolved, object-by-object analysis supports identification of distinct subpopulations and their functional responses over time, providing actionable insights into treatment effects and cellular dynamics that are difficult to capture with widefield imaging. By linking specific spatial and temporal phenotypes to drug mechanisms, resistance pathways, or microenvironmental interactions, the CX3 provides a functional bridge between *in vitro* 3D biology and translational outcomes. In short, object-based analysis on the CX3 does more than quantify structures by uncovering the patterns and behaviors that define true biological response.

To illustrate the utility of this software, mouse hepatic organoids stably expressing Incucyte® Nuclight Orange were monitored over time. In this culture there were a mix of expressing and non-expressing organoids which can be identified using object-by-object software based on fluorescence characteristics (Figure 5A). Segmented organoids were also assessed for their darkness and area as a measure of cell growth. The data demonstrates that both the orange expressing and non-expressing subpopulations show similar growth kinetics over 5 days culture. The images illustrate the identified subpopulations over time.

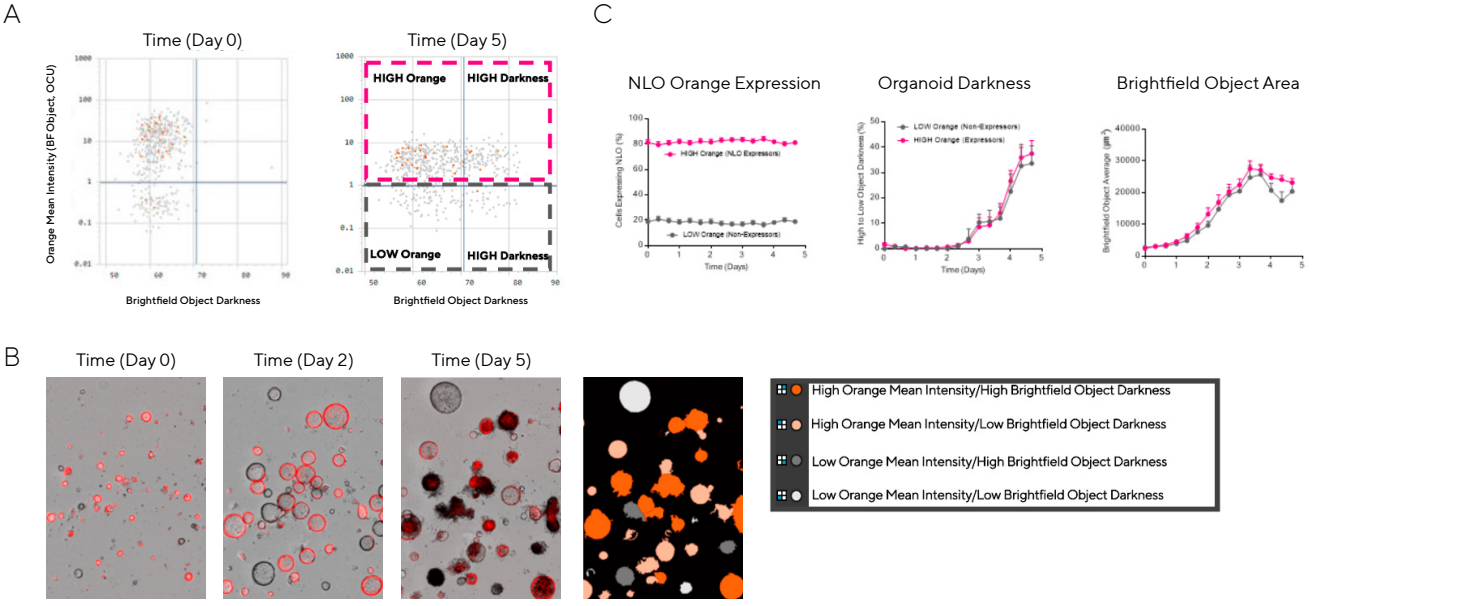


Figure 5. Comparison of Mouse Hepatic Organoid (MHO) Growth Kinetics in Orange Expressing and Non-Expressing Subpopulations. MHO expressing Incucyte® Nuclight Orange (NLO) were cultured for 5 days and imaged in Brightfield, Phase, and Orange confocal fluorescence modes using the Incucyte® Organoid Analysis Module. Segmented brightfield objects were further classified using the 3D Object Classification Software Module. (A) Scatter plots show gating of MHO populations based on Brightfield Object Darkness and Orange Mean Intensity. (B) Representative images (Days 0, 2, and 5) illustrate morphological and fluorescence changes over time, with segmentation masks visualizing the defined subsets. (C) Classification of organoids into HIGH and LOW Orange subsets revealed ~80% NLO expressors and ~20% non-expressors. Comparable collapse kinetics and growth trends were observed between both groups, with peak organoid size between 3–4 days in culture.

Summary and Conclusion

Complex 3D biological models continue to play an increasingly important role in understanding disease mechanisms and evaluating therapeutic candidates. Their structural complexity and physiological relevance demand imaging tools that can resolve the spatial and temporal features that define cellular behavior in these systems.

Confocal imaging provides robust, depth-resolved, and time-dependent data essential for drug development using these complex cell models. It enhances contrast and reduces signal overlap, enabling precise detection of cell health, proliferation, and function, which widefield systems often miss. This clarity is crucial for distinguishing genuine responses from artifacts.

The Incucyte® CX3 Live-Cell Analysis System addresses these needs by integrating confocal imaging directly within the incubator environment. This combination of continuous, non-perturbative acquisition and depth-resolved optical sectioning provides researchers with a clearer and more accurate view of cell development and treatment response across time.

The system supports higher-throughput applications with its capacity for six plates and automated acquisition, making it ideal for long-term studies such as concentration-response experiments, immune-tumor profiling, and panel screening across multiple 3D models. Its scalability across 96- or 384-well plates allows screening-level studies to maintain the biological complexity of models without sacrificing efficiency. Continuous, non-perturbative imaging captures the full kinetics of cellular responses, enhancing reproducibility and providing comprehensive insights beyond isolated endpoints. These capabilities promote broader use of 3D assays in discovery programs, increasing the translational relevance of in vitro findings.

By delivering higher contrast, cleaner signals, and reliable optical section data, the CX3 reduces interpretation uncertainty and bolsters confidence in biological readouts. This clarity makes treatment-dependent changes more detectable, minimizing false negatives and supporting decisive actions in discovery workflows.

The Incucyte® CX3, equipped with automated workflows and integrated analysis tools, empowers researchers to explore complex cell systems with improved reproducibility, deeper mechanistic insights, and greater relevance to in vivo biology. As imaging technologies advance, platforms like the Incucyte® CX3 system will influence the next generation of discovery workflows, helping translate in vitro findings into meaningful therapeutic progress by delivering clarity from complexity.

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