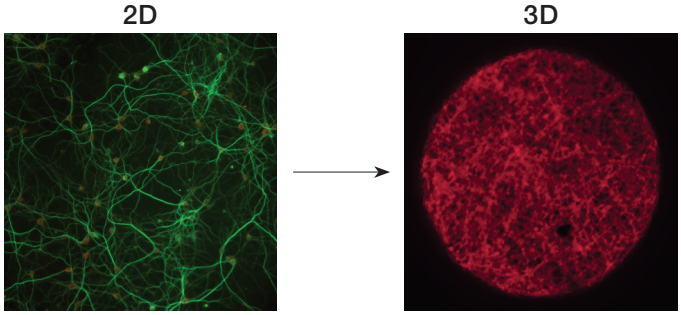
High-throughput fluorescence imaging and analysis of spheroids

Tips, tricks, and tools to efficiently grow, stain, image, and analyze 3D cell cultures

Introduction

As cells do not grow in isolation, traditional two-dimensional (2D) cell culture monolayer models lack physiologically relevant environmental conditions and can have drastic differences in terms of their physical and biochemical features, compared to intact biological systems. Since more physiologically relevant animal studies are time consuming and expensive, there is a need for experimental models that span the gap between *in vitro* cell-based assays and *in vivo* animal studies. To satisfy this need, researchers have been turning to three-dimensional (3D) organoid and tumor spheroid systems that mimic an *in vivo* setting while maintaining the ease of manipulation of cell-based assays. These 3D model systems have several features that allow them to more closely resemble the environment of cells in an intact organism, including cell-to-cell interactions, cell-to-matrix interactions, relevant gene expression profiles, and gradients of oxygen, nutrients, and metabolites [1] (Figure 1). Because 3D spheroids share features with tumors, such as functionally different zones with apoptotic or necrotic regions in the core and proliferative cells along the periphery, they are an excellent model for cancer biology and drug discovery assays. Their features allow better investigation of chemoresistance in the core of tumors to improve drug delivery, along with more relevant drug screening and pharmacology assays [2-4].



	2D monolayer	3D spheroid
Cellular interactions	Cell to cell	Cell to cell, cell to extracellular matrix
Cellular activity	Cellular adhesion, proliferation, and modified genes	Proliferative ring; apoptotic or necrotic core
Microenvironment	Relatively uniform	Gradients of oxygen, metabolites, and nutrients

Figure 1. Comparison of 2D and 3D cellular models. Working in 3D systems involves the formation of spheroids. Spheroids are aggregates of cells that can be either grown in suspension, encapsulated, or grown on top of a 3D matrix. Compared to 2D cell models, 3D spheroids have cellular interactions, zones of cellular activity, and microenvironments that more closely resemble *in vivo* systems, allowing more relevant pharmacology studies and better tumor models.

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3D cell culture and analysis workflow

Using optimal techniques and cell culture reagents, 3D spheroids can easily be grown in the lab and stained with a variety of fluorescent reagents to examine molecular localization and various cellular functions. Fluorescently labeled spheroids can be imaged and analyzed using microplate readers, fluorescence microscopy, or high-content imaging systems. Invitrogen™ and Thermo Scientific™ cell culture systems, reagents, instrumentation, and analysis software are available for a complete 3D cell culture workflow from growth and staining of cells to imaging and quantitation of results (Figure 2). These products enable robust 3D cell cultures with accurate imaging and analysis for more physiologically relevant drug discovery and cancer biology applications.

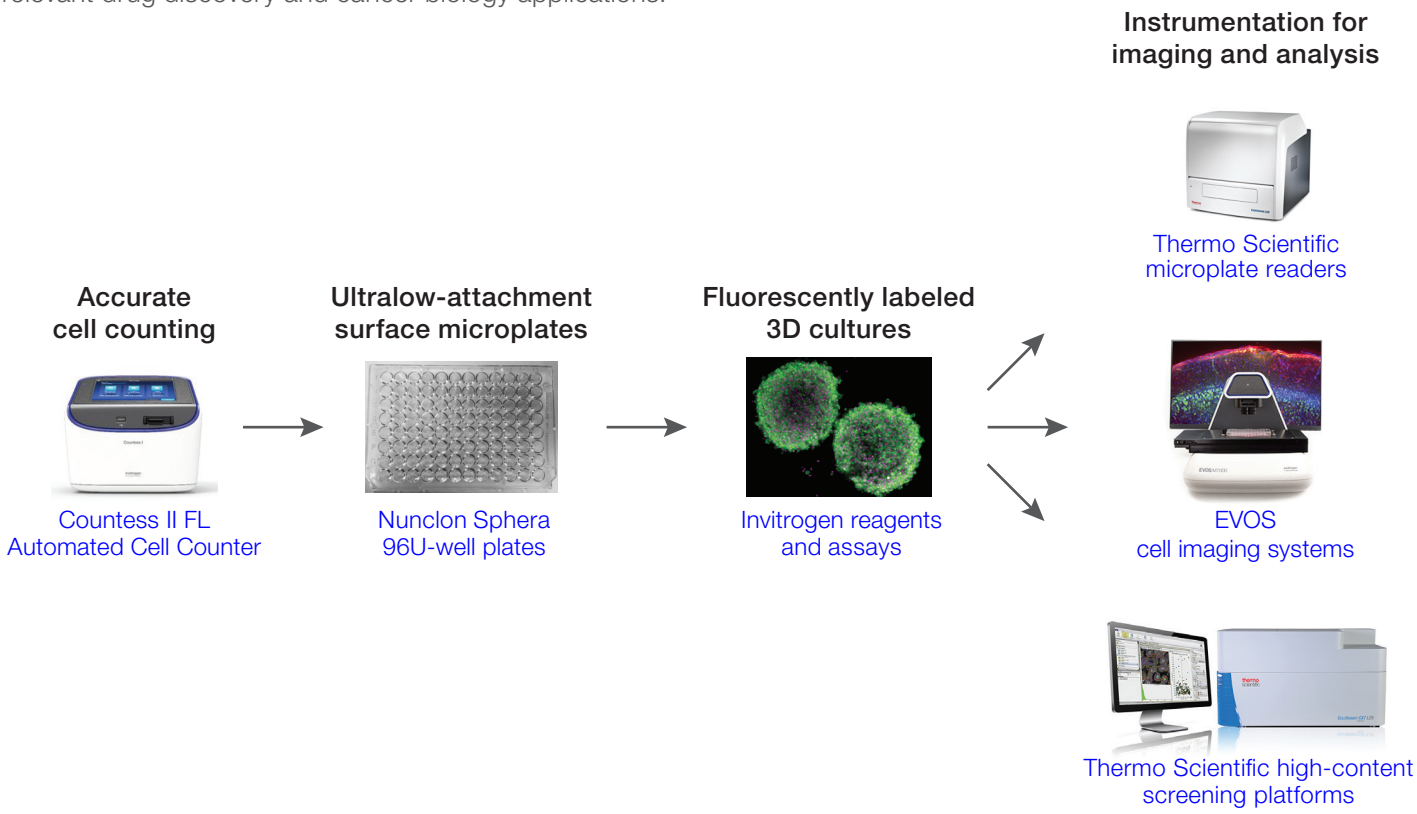

Figure 2. Workflow for spheroid growth, staining, imaging, and quantitation. Accurate cell counting with the Invitrogen™ Countess™ II FL Automated Cell Counter enables cell seeding at the appropriate density for spheroid growth on Thermo Scientific™ Nunclon™ Sphera™ 96U-well plates, which have an ultralow-attachment surface optimal for spheroid growth. Spheroids can then be stained with Invitrogen™ fluorescent reagents and assays followed by imaging and analysis on several fluorescence platforms, including Thermo Scientific™ microplate readers, Invitrogen™ EVOS™ cell imaging systems, and Thermo Scientific™ high-content screening (HCS) platforms.

How to grow spheroids

Although growing cells in a 3D assembly is more challenging than monolayer culturing techniques, spheroid growth of many cell types can easily be accomplished using optimized tools and methods. To efficiently grow spheroids in culture, it is important to seed cells at the appropriate cell density and use cell culture plates that are optimized for 3D growth. These plates have an ultralow-attachment surface compared to traditional tissue culture–treated plates, which encourages cells to grow together in a 3D assembly instead of on a single surface. Nunclon Sphera 96U-bottom plates have an ultralow-attachment, round-bottom surface, which minimizes monolayer cell adhesion and promotes the growth of single spheroids rather than multiple spheroids or satellite 3D cultures, compared to other types of plates (Figure 3). The Countess II FL Automated Cell Counter can be used to accurately count cells so that they are seeded on 3D culture plates at the appropriate density for growing into a 3D spheroid. Growth of cells into 3D cell culture can also be aided by briefly centrifuging the plate after the appropriate number of cells are plated.

Because cells grown in 3D culture are growing together in a free-floating 3D structure instead of attaching to a surface, gentle pipetting is required to change the medium without disturbing or aspirating the spheroid. This can be more easily accomplished by growing the spheroid in a relatively large volume of medium (for instance, 200 µL per well in a 96-well plate) and replacing half the medium every other day rather than removing the entire volume of medium. The use of media and supplements optimized and verified for 3D cell culture also enables spheroids to form more quickly, which can minimize the number of medium changes and allows faster maturation of cells with mature phenotypes. Some cells may also need external factors such as cadherin to grow into a 3D complex.

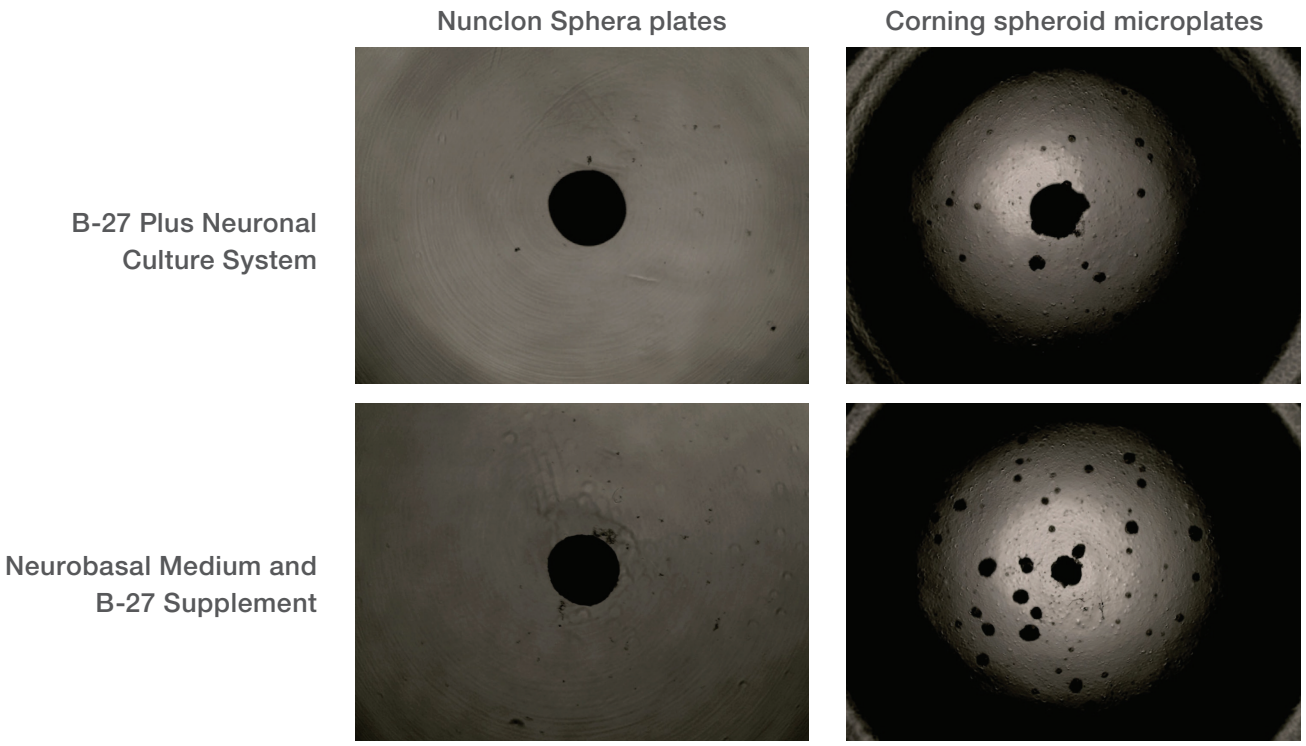

Figure 3. Optimal growth of spheroids on Nunclon Sphera 96U-well plates. Neural stem cells (NSCs) were seeded at 5,000 cells per well on Nunclon Sphera 96U-well plates or Corning™ 96-well Ultra-Low Attachment Spheroid Microplates and grown for 10 days in the Gibco™ B-27™ Plus Neuronal Culture System or Gibco™ Neurobasal™ Medium and B-27™ Supplement. Imaging on an Invitrogen™ EVOS™ XL Core Imaging System showed that NSCs grown on Nunclon Sphera plates formed single, tight neurospheroids while NSCs grown on other plates formed multiple spheroids with satellite cultures.

How to stain spheroids

Spheroids can be stained with a variety of fluorescent reagents to examine localization and abundance of molecules within the 3D cell culture, along with viability, apoptosis, mitochondrial health, and other cellular functions (Table 1). Many fluorescent stains and probes developed for 2D cell culture staining can also be used to stain spheroids. However, because 3D cell cultures are denser than monolayers, concentration and incubation time may need to be optimized to allow stains to fully penetrate into the center of the 3D culture, and the duration of wash steps may need to be increased to reduce background. When immunostaining fixed and permeabilized spheroids, adding 5% DMSO to the antibody staining solution can help reduce background.

Table 1. Fluorescent reagents and assays for spheroid staining and analysis on different detection platforms.

Cellular function	Reagent or assay	Detection platform	Experimental guidelines	
			Concentration*	Incubation time
Apoptosis	CellEvent Green Caspase-3/7	Microplate reader	0.33X	2 hr
		Fluorescence microscopy, high-content screening	1X	1 hr
Mitochondrial health	MitoTracker Orange	Microplate reader	2X	1 hr
		Fluorescence microscopy, high-content screening	1X	30 min–1 hr
Proliferation	Click-iT EdU	Fluorescence microscopy, high-content screening	1X	Standard protocol
Viability	CyQUANT Direct Green	Microplate reader	1X dye, 2X background suppressor	45 min–1 hr
	CyQUANT XTT	Microplate reader	2X	5–8 hr
	PrestoBlue HS	Microplate reader	1X	3–5 hr
	alamarBlue HS	Microplate reader	1X	3–5 hr
	CyQUANT LDH Cytotoxicity Assay	Microplate reader	1X	30 min
	CyQUANT LDH, fluorescence	Microplate reader	1X	10 min
	LIVE/DEAD Viability/Cytotoxicity Kit	Fluorescence microscopy, high-content screening	1X	Standard protocol
Reactive oxygen species	CellROX Deep Red	Fluorescence microscopy, high-content screening	1X	1 hr
Hypoxia	Image-iT Hypoxia Reagent	Fluorescence microscopy, high-content screening	1X	1 hr

* Concentration recommended for 3D staining relative to the manufacturer's recommended working conditions for 2D staining.

Imaging and analysis of spheroids

Once spheroids are stained with the fluorescent reagents of choice, there are a variety of ways to image and analyze them to obtain data from 3D cellular models. Microplate assays can be used to quickly assess the general cell health of the entire spheroid, while more complex imaging and analysis allows optical sectioning to visualize cells within the spheroid core and assess individual cells in the 3D culture. Depending on the type of readout and level of detail needed, fluorescently labeled spheroids can be detected and analyzed with microplate readers, fluorescence microscopes, and high-content screening systems. Poor imaging in the core of a 3D cell culture is often thought to be due to reagent penetration issues; furthermore, light scattering can cause significant limitations in spheroid imaging. Selection of the optimal imaging and analysis system enables fluorescence detection throughout the entire spheroid, increasing the quality of 3D culture images and data.

Tissue clearing reagents, such as Invitrogen™ CytoVista™ 3D Cell Culture Clearing Reagent, can improve imaging of 3D cultures. Treatment of spheroids with this reagent improves visualization of cells in the core of 3D spheroids to enable better fluorescence detection and analysis of cells inside the spheroid (Figure 4).

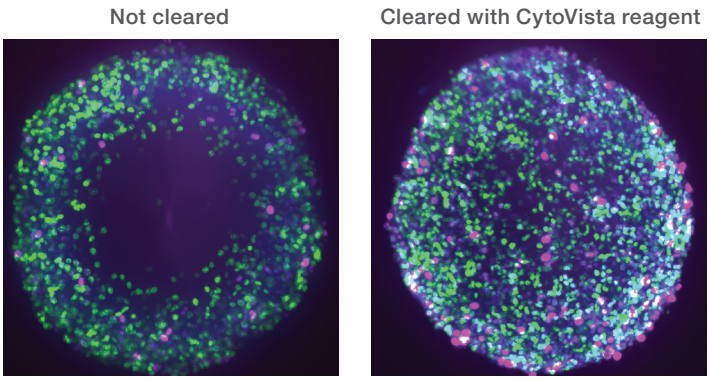

Figure 4. Improved imaging and resolution of a spheroid core cleared with CytoVista 3D Cell Culture Clearing Reagent. A549 spheroids were grown on Nunclon Sphera 96U-well plates at a density of 5,000 cells per well. Spheroids were pulsed with 10 µM 5-ethynyl-2′-deoxyuridine (EdU) for 1 hr. Spheroids were fixed and permeabilized, and then labeled with the Invitrogen™ Click-iT™ EdU Alexa Fluor™ 488 HCS Assay following the kit protocol. Spheroids were also stained with Invitrogen™ Hoechst 34580 and Ki67 mouse monoclonal antibody that was labeled using the Invitrogen™ Zip Alexa Fluor™ 647 Rapid Antibody Labeling Kit. The spheroids were then treated with CytoVista 3D Cell Culture Clearing Reagent for 1 hour or left untreated. The spheroids were imaged with the Thermo Scientific™ CellInsight™ CX7 LZR High Content Analysis Platform using confocal mode. The image is a maximum-intensity projection of 100 optical sections of 2 µm each.

Microplate readers

Microplate assays can be used to quickly assess cell viability and health as a preliminary measurement before downstream applications such as drug characterization. Spheroids require higher drug concentrations for effectiveness, resulting in different IC_{50} values compared to those obtained for the same drug in 2D cultures. IC_{50} values obtained from drug-treated spheroids analyzed by microplate readers and high-content analysis systems are similar, indicating that microplate readers can be used to quickly screen spheroid drug responses and provide similar data as cell-by-cell quantitation (Figure 5).

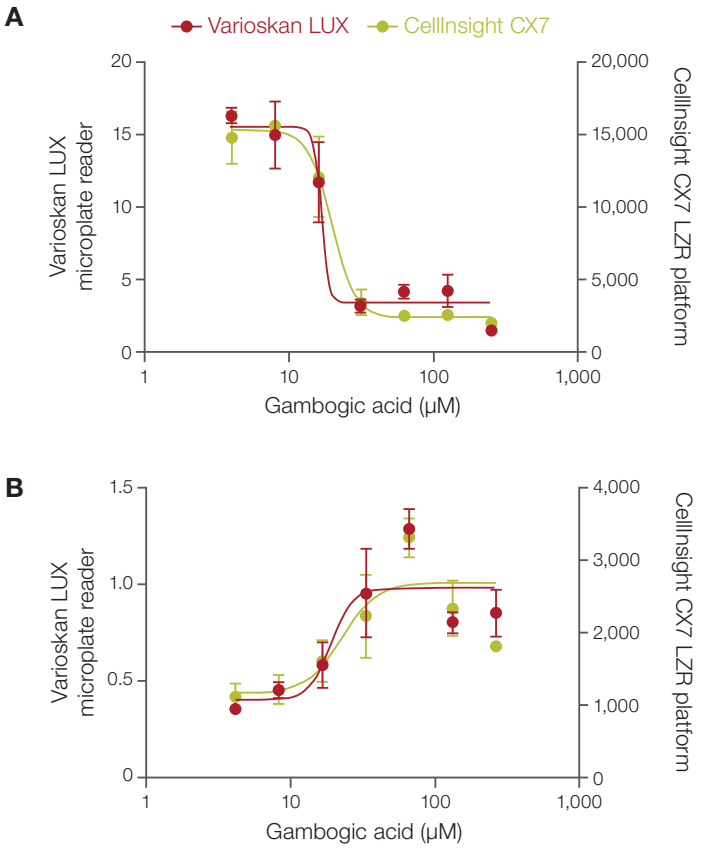

Figure 5. Drug dose response in 3D spheroids. A549 spheroids were treated with various concentrations of gambogic acid for 48 hours, then stained using the **(A)** Invitrogen™ CyQUANT™ Direct Cell Proliferation Assay or **(B)** Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent for analysis of apoptosis. Fluorescence (500 nm excitation, 530 nm emission) was read on the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader or the CellInsight CX7 LZR High Content Analysis Platform. For each reagent, similar IC_{50} values were obtained on both the microplate reader and high-content analysis platform, indicating that fluorescence-based assays can be analyzed with microplate readers for initial drug discovery questions.

Fluorescence microscopy

When imaging 3D cultures with fluorescence microscopy, optimization of microscope settings and the use of advanced features can improve image quality and allow better visualization into the spheroid core. Selection of the appropriate objective improves axial resolution and focus, while microscopes with z-stacking capabilities can be used to image throughout entire spheroids. EVOS cell imaging systems are high-performance, easy-to-use imaging systems with z-stacking capabilities that can be used to produce high-resolution images of 3D cultures. These images can then be analyzed on an image analysis platform such as Invitrogen™ Celleste™ Image Analysis Software for quantitation of spheroid size and general intensity measurements of the entire spheroid (Figure 6).

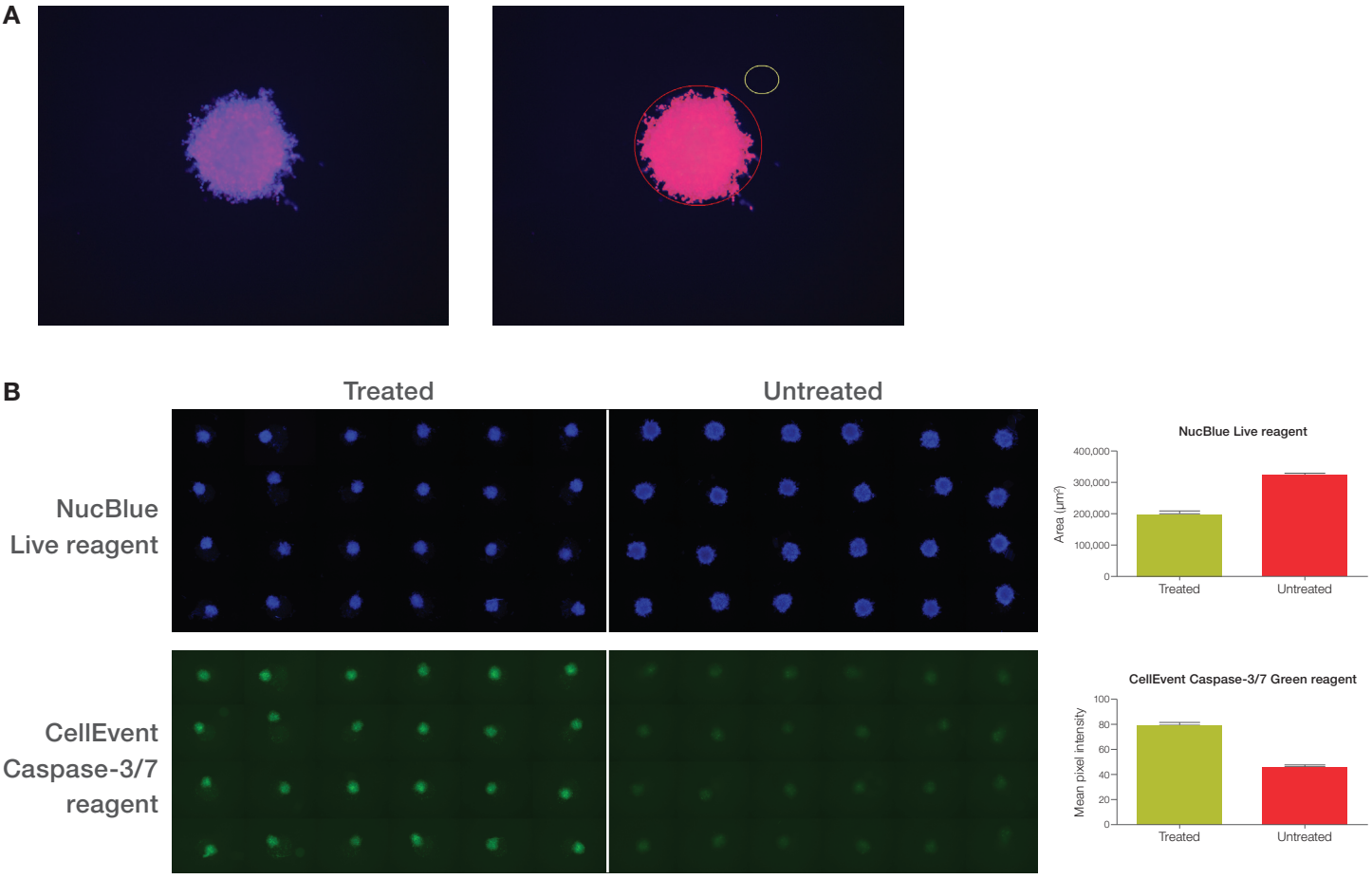

Figure 6. Spheroid imaging on the Invitrogen™ EVOS™ FL Auto 2 Imaging System and segmentation with Celleste Image Analysis Software. **(A)** A549 cell spheroids were cultured in Nunclon Sphera 96U-well plates and subsequently labeled with Invitrogen™ NucBlue™ Live, MitoTracker™ Orange, and CellEvent Caspase-3/7 Green reagents, followed by imaging on the EVOS FL Auto 2 Imaging System using an Olympus™ 4x super-apochromat objective with DAPI, RFP, and GFP filter cubes. Images were imported into Celleste 4.1 software for automated analysis of spheroid morphology and staining intensity using smart segmentation functionality, shown as red (object of interest) and yellow (background) regions of interest. Upon defining the two regions, Celleste 4.1 software is able to automatically detect objects and successfully segment the spheroid versus background. **(B)** Representative montage images showing A549 spheroids that were treated with 40 µM niclosamide or DMSO control. Half the plate was treated, and the other half was untreated. Figures show that treated spheroids are smaller in size, as determined by measuring the area defined by staining with NucBlue Live reagent, and that they have an increase in apoptotic activity, as determined by staining with CellEvent Caspase-3/7 Green reagent. Data were generated by segmenting the spheroids using the smart segmentation inputs defined in (A) and applying the analysis to the 48 wells shown in the representative images. Each bar represents the mean ± SEM for 24 spheroids.

High-content screening

High-content screening offers the benefits of rapid and high-throughput cell imaging and analysis of large cell populations with robust quantitation and statistics. The CellInsight CX7 LZR High Content Analysis Platform has several features that make it an ideal platform for high-content imaging and analysis of 3D cell cultures. This system incorporates high-resolution imaging and the ability to obtain z-stacks, along with a low scanning time and confocal mode. Its laser-based excitation source results in reduced light scatter, deeper penetration, and improved signal-to-noise ratios, compared to LED-based excitation sources.

Thermo Scientific™ HCS Studio™ 3.1 Cell Analysis Software enables cell-by-cell analysis of these high-resolution 3D culture images. The software can segment individual cells within the 3D spheroid and measure cell-by-cell intensity and other features to provide detailed analysis of each cell within the 3D structure (Figure 7).

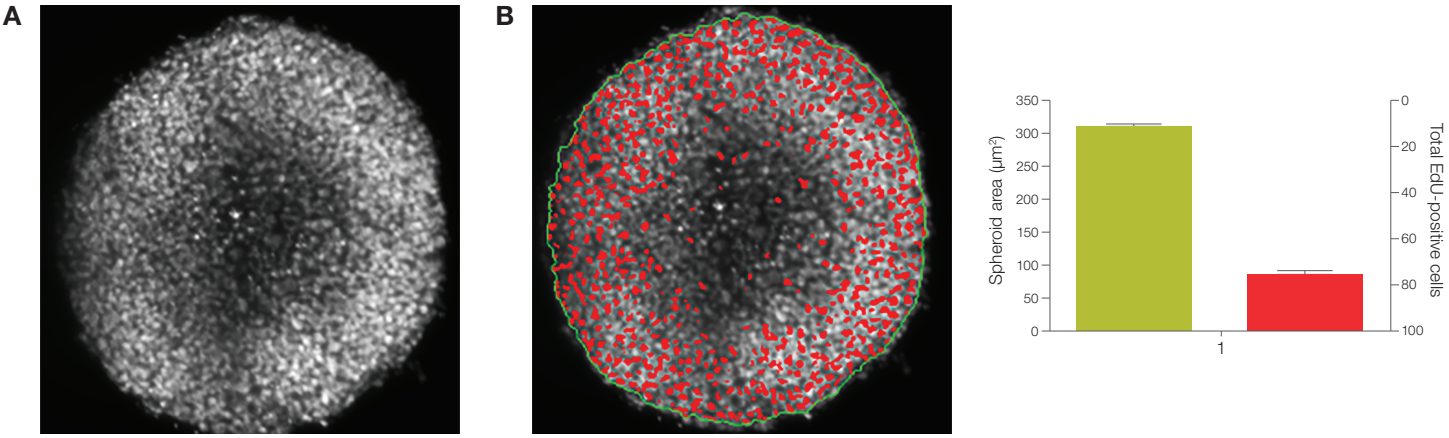

Figure 7. Quantitation of cell proliferation in spheroids, with high-content screening. A549 cells were plated at a density of 5,000 cells per well on a Nunclon Sphera 96U-well microplate and incubated for 24 hours in a CO_2 incubator. EdU was added to a final concentration of 10 µM and incubated for 1 hr. The spheroids were then fixed with 4% formaldehyde and permeabilized with 0.25% Triton™ X-100 surfactant. The spheroids were then stained for EdU using the Click-iT EdU Alexa Fluor 488 HCS Assay, following the kit protocol. **(A)** The plate was imaged on the CellInsight CX7 LZR High Content Analysis Platform with a 4x objective using confocal mode. The image is a maximum-intensity projection of 200 optical z-slices of 1 µm each. **(B)** Quantitation was done with HCS Studio software using the Morphology Explorer bio-application. The spheroid was segmented as one object, and EdU-positive cells were counted as spots within the spheroid.

Conclusion

With optimal cell culture reagents, robust fluorescent reagents and assays, and high-performance fluorescence imaging and detection systems, switching from 2D to 3D cell culture can be easily accomplished even in standard laboratory settings. Because 3D spheroids have a cellular environment and other features that more closely resemble tumors and *in vivo* models, research on these cultures can provide more relevant data and findings that apply to intact biological systems, enhancing research in drug discovery, cancer biology, and other critical areas.

References

1. Baker BM, Chen CS (2012) Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. *J Cell Sci* 125(Pt 13):3015–3024.
2. Huang BW, Gao JQ (2018) Application of 3D cultured multicellular spheroid tumor models in tumor-targeted drug delivery system research. *J Control Release* 270:246–259.
3. Patel NR, Aryasomayajula B, Abouzeid AH et al. (2015) Cancer cell spheroids for screening of chemotherapeutics and drug-delivery systems. *Ther Deliv* 6(4):509–520.
4. Fang Y, Eglen RM (2017) Three-dimensional cell cultures in drug discovery and development. *SLAS Discov* 22(5):456–472.

Ordering information

Product	Cat. No.
Countess II FL Automated Cell Counter	AMQAF1000
Countess Cell Counting Chamber Slides	C10228
Nunclon Sphera 96U-well plates	174925
EVOS XL Core Imaging System	AMEX1000
Varioskan LUX Multimode Microplate Reader	VL0LATD2
CellInsight CX7 LZR High Content Analysis Platform	CX7A1110LZR
EVOS M7000 Cell Imaging System	AMF7000
Celleste Image Analysis Software	AMEP4816
CyQUANT Direct Cell Proliferation Assay	C35011
CellEvent Caspase-3/7 Green Detection Reagent	C10423
NucBlue Live ReadyProbes Reagent	R37605
Click-iT EdU Alexa Fluor 488 HCS Assay	C10350
B-27 Plus Neuronal Culture System (contains Neurobasal Plus Medium and B-27 Plus Supplement)	A365340
Zip Alexa Fluor 647 Rapid Antibody Labeling Kit	Z11235
Hoechst 34580	H21486
CytoVista 3D Cell Culture Clearing Reagent	V11315
MitoTracker Orange CMTMRos	M7510
CyQUANT XTT Cell Viability Assay	X12223
PrestoBlue HS Cell Viability Reagent	P50200
alamarBlue HS Cell Viability Reagent	A50100
CyQUANT LDH Cytotoxicity Assay	C20300
CyQUANT LDH Cytotoxicity Assay, fluorescence	C20302
LIVE/DEAD Viability/Cytotoxicity Kit	L3224
CellROX Deep Red	C10422
Image-iT Green Hypoxia Reagent	I14834
Olympus 4x Objective, super-apochromat, LWD	AMEP4752
HCS Studio 3.1 Cell Analysis Software	thermofisher.com/hcs

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