Analysis of cancer spheroids through high-throughput screening assays

Introduction

Cancer cells grown as spheroids resemble human tumors more closely than cells grown in monolayers, with respect to morphology, structural complexity, phenotype, and sensitivity to chemotherapeutics. Being more physiologically relevant model systems, they can be more predictive of drug profiling and cytotoxicity. So, early screens of drugs have become more dependent on 3D cell culture. In recent years, tumor-derived spheroids have been utilized to optimize cancer therapeutics for ovarian and hepatocellular carcinoma [**1**,**2**,**3**]. However, there are some challenges to using spheroids for drug screening: primarily, the number of spheroids per well, and the shape and size of spheroids, need to be uniform in order to reduce variability between replicates. To address this challenge, we compiled tips and tricks on how to generate uniform and reproducible cancer spheroids for high-throughput screening (HTS) assays in an application note titled "**Generation of cancer spheroids—tips and tricks**". Additionally, we have outlined a workflow for robust 3D cancer spheroid generation in Figure 1.

Graphical workflow of 3D cancer spheroid generation

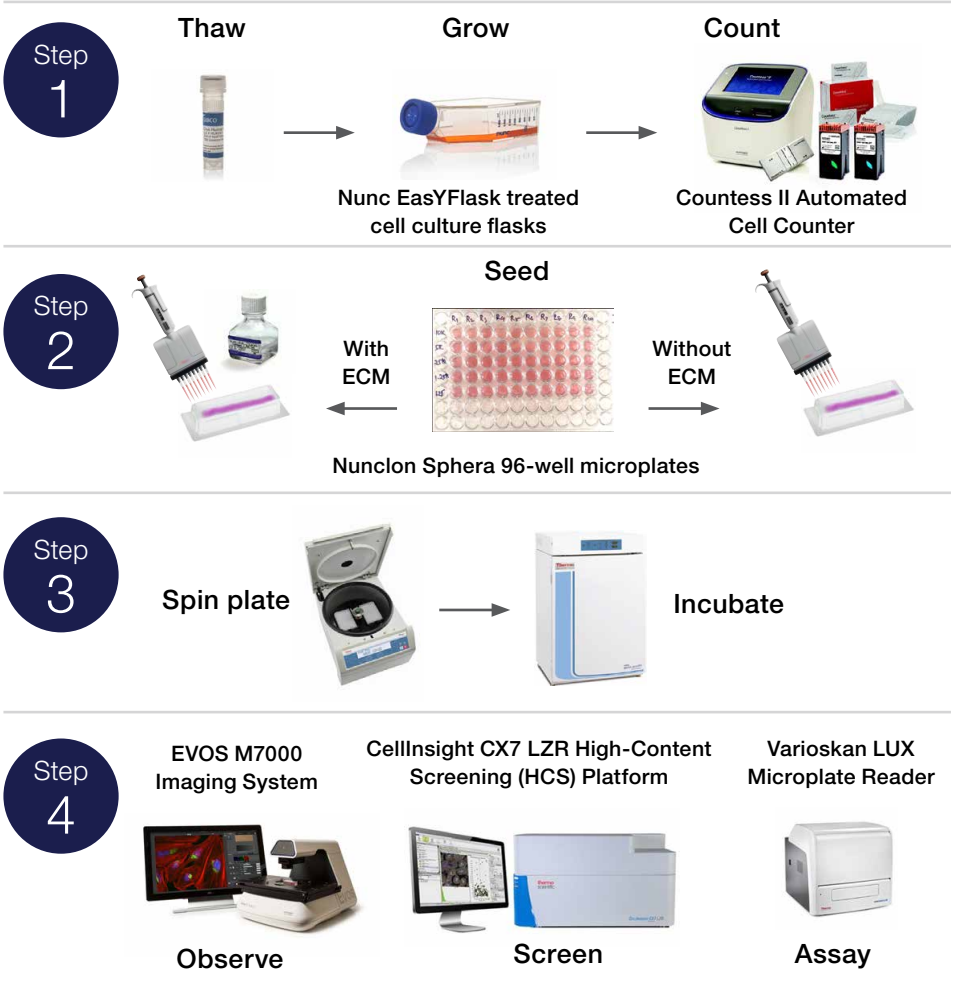

Figure 1. Schematic representation of the process of spheroid generation on Thermo Scientific™ Nunclon™ Sphera™ plates.

Another challenge in working with spheroids is determining penetration of drugs to optimize treatment times. Moreover, the use of spheroids can complicate experimental design and interpretation, but this can be overcome by using the right kinds of reagents, equipment, and protocols. Here we outline different kinds of HTS assays that can be performed on cancer spheroids to assess drug response. We also provide some useful guidelines for handling spheroids and acquiring data to get the most meaningful results. All spheroids were generated on Nunclon Sphera plates using the appropriate Gibco™ cell culture medium.

Assays with plate reader–based readouts

Cell viability and cytotoxicity assays using Invitrogen™ PrestoBlue™ HS Cell Viability Reagent

This straightforward assay utilizes resazurin as a cell health monitor. Upon entering healthy cells, resazurin is reduced in the mitochondria to resorufin, resulting in fluorescence (Ex/Em 560/590 nm). Using this assay, we compared the response of cells in 2D and 3D cultures to doxorubicin, a chemotherapeutic agent. Two different types of cancer cells (HepG2 and PANC-1) were considered. Spheroids and monolayers were treated with doxorubicin either 4 (HepG2) or 7 days (PANC-1) after plating and allowed to incubate for 72 hours. PrestoBlue HS reagent was then added 1:10 (v/v) to the spent medium, and spheroids were incubated at 37°C for 6 hours.

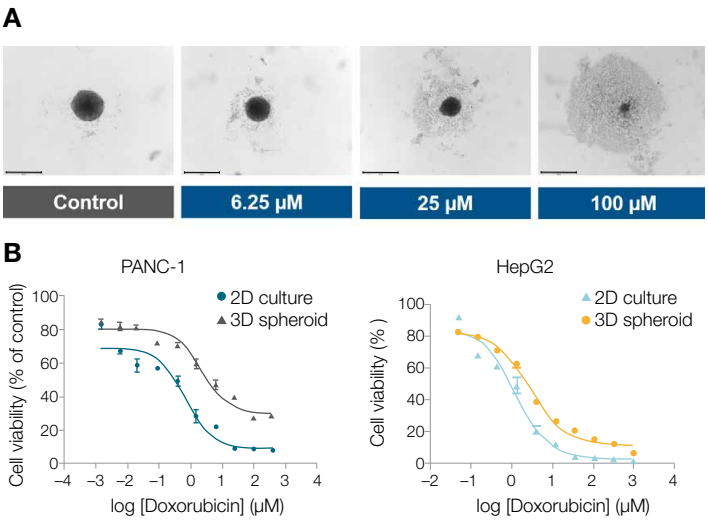

Figure 2. Morphology and effectiveness of doxorubicin treatment on spheroids in 2D and 3D cultures. (A) Morphology of control and doxorubicin-treated PANC-1 spheroids 72 hours posttreatment. Images were captured using the **Thermo Scientific™ EVOS™ M7000 Imaging System** under a 4x objective. Scale bar: 650 µm. **(B)** Dose response curves for doxorubicin-treated PANC-1 (left) and HepG2 (right) spheroids in 2D and 3D cultures.

Following this, high-throughput readouts were obtained using the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader. We recommend taking a top read for homogeneity between experimental repeats. Nonlinear regression analysis was performed for variable slope of log (inhibitor) vs. response to calculate the IC_{50} using GraphPad Prism 5.01. As seen with PANC-1 (Figure 2A), doxorubicin treatment caused disintegration of spheroids with increasing dose, indicating cytotoxicity. For both cell lines, the IC_{50} of doxorubicin for 3D culture was at least twice that for the 2D culture (Figure 2B), suggesting increased sensitivity of 2D cultures towards the drug.

Analyzing PSA levels using the Invitrogen™ PSA (Total)/KLK3 Human ELISA Kit

Prostate-specific antigen (PSA) in serum is a known biomarker for prostate cancer diagnosis. The PSA (Total)/KLK3 Human ELISA Kit has been successfully used to detect PSA in cell culture supernatant from 2D culture [**4**]. Using the manufacturer's instructions, we compared PSA secretion in 2D and 3D cell cultures. We chose the LNCaP cell line that expresses the *KLK3* gene (which in turn encodes PSA) endogenously. Medium from PC-3 cells, which do not produce PSA endogenously, was used as a negative control. LNCaP monolayers and spheroids were treated with 2 nM dihydrotestosterone (DHT, which enhances PSA expression) or 80 µM cisplatin (represses PSA expression) 4 days after plating, and incubated for 48 hours. Culture supernatant was collected, diluted 1:20 in diluent buffer, and assayed for secreted PSA using the PSA (Total)/KLK3 Human ELISA Kit and the Varioskan LUX Multimode Plate Reader for colorimetric reading. The colorimetric readings were used to calculate relative PSA levels according to the kit instructions.

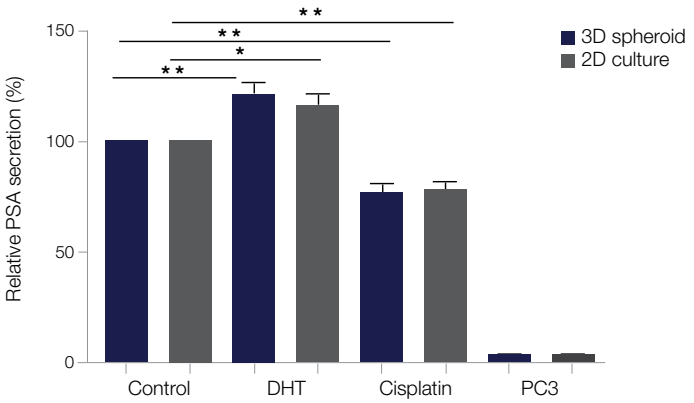

Figure 3. Quantification by ELISA of secretion of PSA following treatment with DHT and cisplatin. Error bars denote standard error of the mean. N = 2. *P < 0.01 and **P < 0.001 for difference from untreated control by one-way analysis of variance (ANOVA).

DHT treatment resulted in 17% and 21% increases in PSA secretion in 2D and 3D culture, respectively, while treatment with cisplatin reduced endogenous PSA secretion by 22–23% (Figure 3). However, 3D culture did not show any major difference in resting or induced PSA levels from 2D culture. This exemplifies how conditioned medium from spheroids can be used for high-throughput non–cell-based assays. In fact, using appropriate readouts, multiplexing of assays can also be performed.

Assays with image-based readouts

Cell viability/cytotoxicity assay using Invitrogen™ LIVE/DEAD™ kit

The LIVE/DEAD kit is a two-color assay that measures cell viability based on plasma membrane integrity and esterase activity. It discriminates live cells from dead cells by staining live cells with Invitrogen™ calcein AM, which is converted to green-fluorescent calcein by intracellular esterase activity, and dead cells with red-fluorescent ethidium homodimer 1 (EthD-1), indicating loss of plasma membrane integrity. After 1 day in culture, SKOV-3 spheroids were treated with various concentrations of the chemotherapeutic drug paclitaxel for 72 hours, followed by incubation with 1 µM each of calcein AM and EthD-1 at 37°C for 3 hours. Following this, spheroids were washed by exchanging half of the medium with 1X PBS, then imaged. We found that exchanging the medium gently from the sides of the wells works better than centrifuging the plates and helps the spheroids stay at the center of the wells, thus aiding in image acquisition (Figure 4A). Spheroids were autofocused using the DAPI channel (they were incubated with **Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent** along with calcein AM and EthD-1 staining), and the centered, maximum-intensity image projection was used to capture the z-stacks. Images were captured using the **Thermo Scientific™ CellInsight™ CX7 High-Content Screening Platform** and analyzed using the cell viability tool on Thermo Scientific™ HCS Studio Cell Analysis Software 4.0. Calcein fluorescence values in the treated samples were normalized to those of the control samples to calculate percentage of viable cells. Values were plotted against paclitaxel concentration using GraphPad Prism software. Increasing paclitaxel concentration led to concomitant reduction in cell viability (Figure 4B).

Apoptosis assay using Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent

The reagent is a four–amino acid peptide (DEVD) conjugated to a nucleic acid–binding dye. The dye is nonfluorescent unless DEVD is cleaved by active caspase-3/7. Following DEVD cleavage, the dye is able to bind to DNA and give a fluorescence signal, providing a means to detect cells undergoing caspase-3/7–dependent apoptosis. MDA-MB-231 spheroids were formed using collagen I as previously **described**, and on day 4 treated with various concentrations of the caspase-dependent, apoptosis-inducing drug etoposide for 72 hours. Spheroids were then incubated with 2 µM of the CellEvent Caspase-3/7 Green Detection Reagent and 1 drop of Invitrogen™ NucBlue™ reagent per milliliter of PBS at 37°C for 2 hours. If PBS is used at this stage, spheroids do not require additional washing. Images were captured on the CellInsight CX7 High-Content Screening Platform under a 4x objective in confocal mode and analyzed using the spot measurement tool of HCS Studio software 4.1.

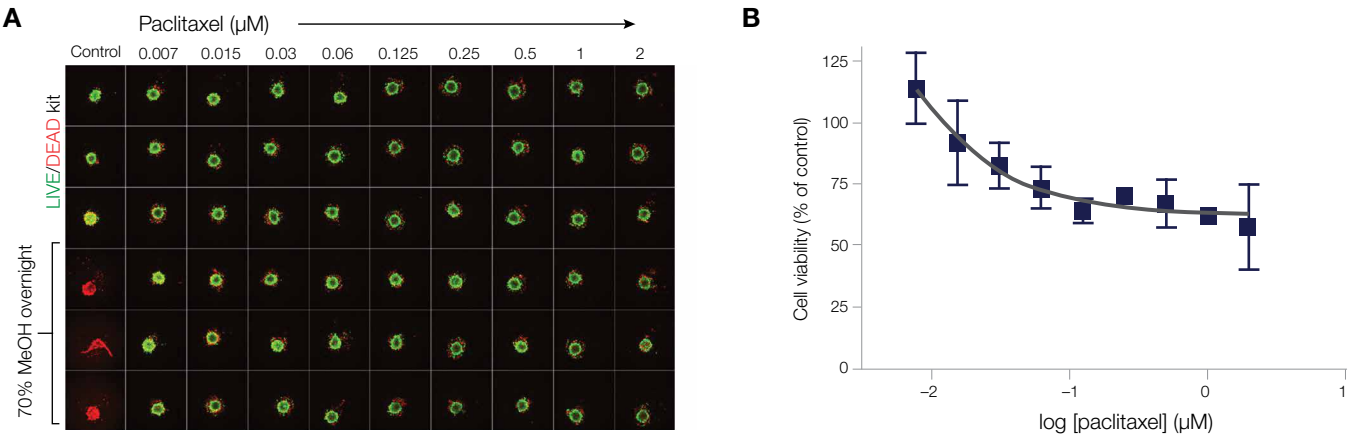

Figure 4. Cell viability assay analysis. (A) Image montage showing LIVE/DEAD staining of SKOV-3 spheroids following treatment with paclitaxel. Images were acquired using the CellInsight CX7 HCS Platform under a 4x objective and in confocal mode. Cells treated with 70% methanol (to kill the cells) in the specified wells served as a negative control for the assay. **(B)** Plot of percent viability of cells with increasing paclitaxel concentrations. The values obtained using HCS Studio software were plotted in GraphPad Prism software and were fit to scale using nonlinear regression. N = 2.

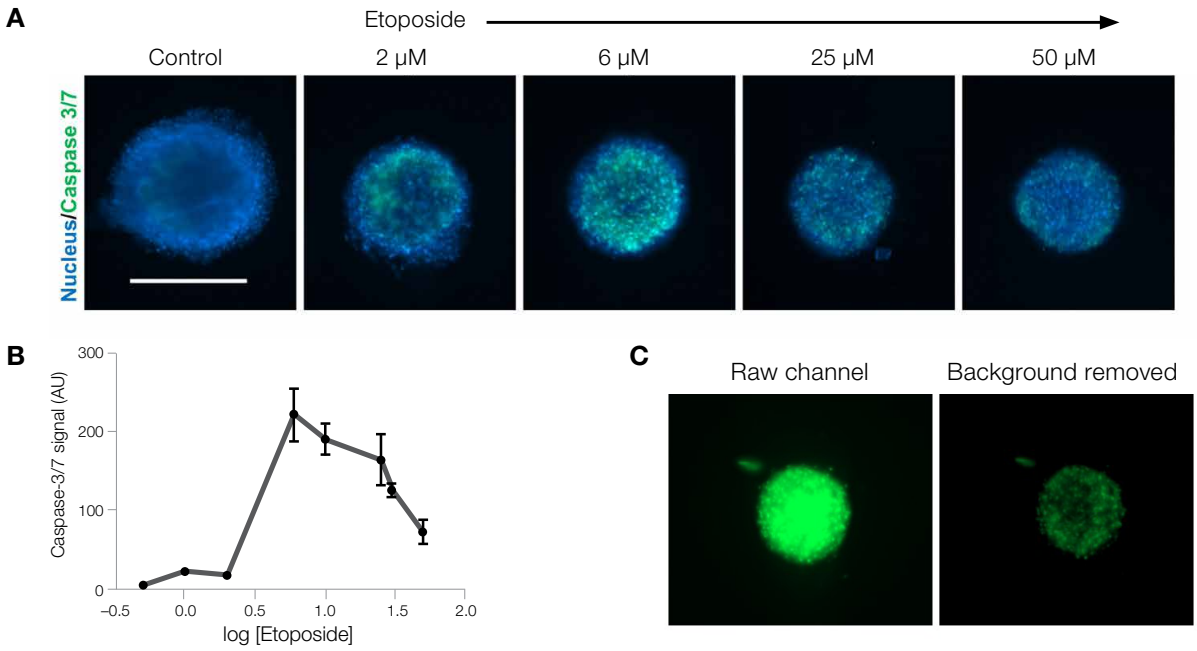

Figure 5. Apoptosis assay analysis. (A) Representative images of control and etoposide-treated MDA-MB-231 spheroids. Scale bar: 500 µm. **(B)** Plot of caspase-3/7 signal intensity against increasing concentrations of etoposide. Six spheroids were considered for every treatment concentration. The plot was generated using GraphPad Prism software from the data obtained from HCS Studio software. Error bars represent standard deviation; N = 2. **(C)** Representation of raw channel (left) and background-corrected channel image (right) for MDA-MB-231 spheroids.

Compared to the control, there was an increase in caspase-3/7 signal with increasing etoposide concentration. However, beyond 6 µM etoposide, the caspase-3/7 signal decreased gradually, possibly owing to an increase in cell death (Figure 5A, B). Another point to note is that the extracellular matrix created background in staining, but using the background removal function for the green channel in HCS Studio software removed it (Figure 5C).

Cell proliferation assay using the Invitrogen™ ClickiT™ EdU Cell Proliferation Kit

This kit uses "click" chemistry to detect cells undergoing new DNA synthesis. T-47D cells were allowed to form spheroids for 24 hours, after which they were treated with 100 nM colchicine, an inhibitor of the mitotic phase of the cell cycle. After approximately 30 hours of treatment, 50% of the spent medium was exchanged with fresh medium containing 20 µM EdU and incubated overnight at 37^{0}C. Proliferating cells that had incorporated EdU were detected using the Click-iT EdU Cell Proliferation Kit with slight changes in the manufacturer's protocol. Briefly, cells were fixed in 3.7% Thermo Scientific™ Pierce™ Formaldehyde for 30 minutes and permeabilized with 0.25% Thermo Scientific™ Triton™ X-100 detergent for 1 hour, followed by incubation with Click-iT EdU dye detection cocktail overnight (as opposed to 30 minutes at room temperature as stated in the kit instructions).

Due to multiple washes involved in the protocol, it is possible that spheroids get dislodged from the center of the well. As a result, they don't always fall completely in the path of light. This gives erroneous readings and variability between replicates. Thus, visualizing the spheroids followed by analysis gives more meaningful data. An example is shown in Figure 6A. Here, both spheroids have been dislodged from the center of the well, but the spheroid in the right panel (shown with arrow) is only partially captured in the field of view. Hence it was excluded from the analysis. Also, small spheroids (200–400 µm) had to be used in the assay to capture most of the spheroids on the Thermo Scientific™ CellInsight CX7 and CX7 LZR HCS Platforms. However, this challenge has been resolved with a new software technology, Thermo Scientific™ EurekaScan™ Finder. EurekaScan Finder has a "seek and find" feature for the CellInsight CX7 LED and LZR HCS Platforms aimed at accelerating discovery by automating the identification and capture of irregularly seeded biological samples, including spheroids, at progressively higher magnifications. With the EurekaScan Finder feature applied, specimens are identified during low-magnification "seek" operations and, once "found", efficiently scanned at higher magnifications for optimal resolution. EurekaScan Finder allows scientists to first identify samples using low magnification across large surface areas, capture them at intermediate magnification, then evaluate them for rare events or improved resolution at higher magnifications.

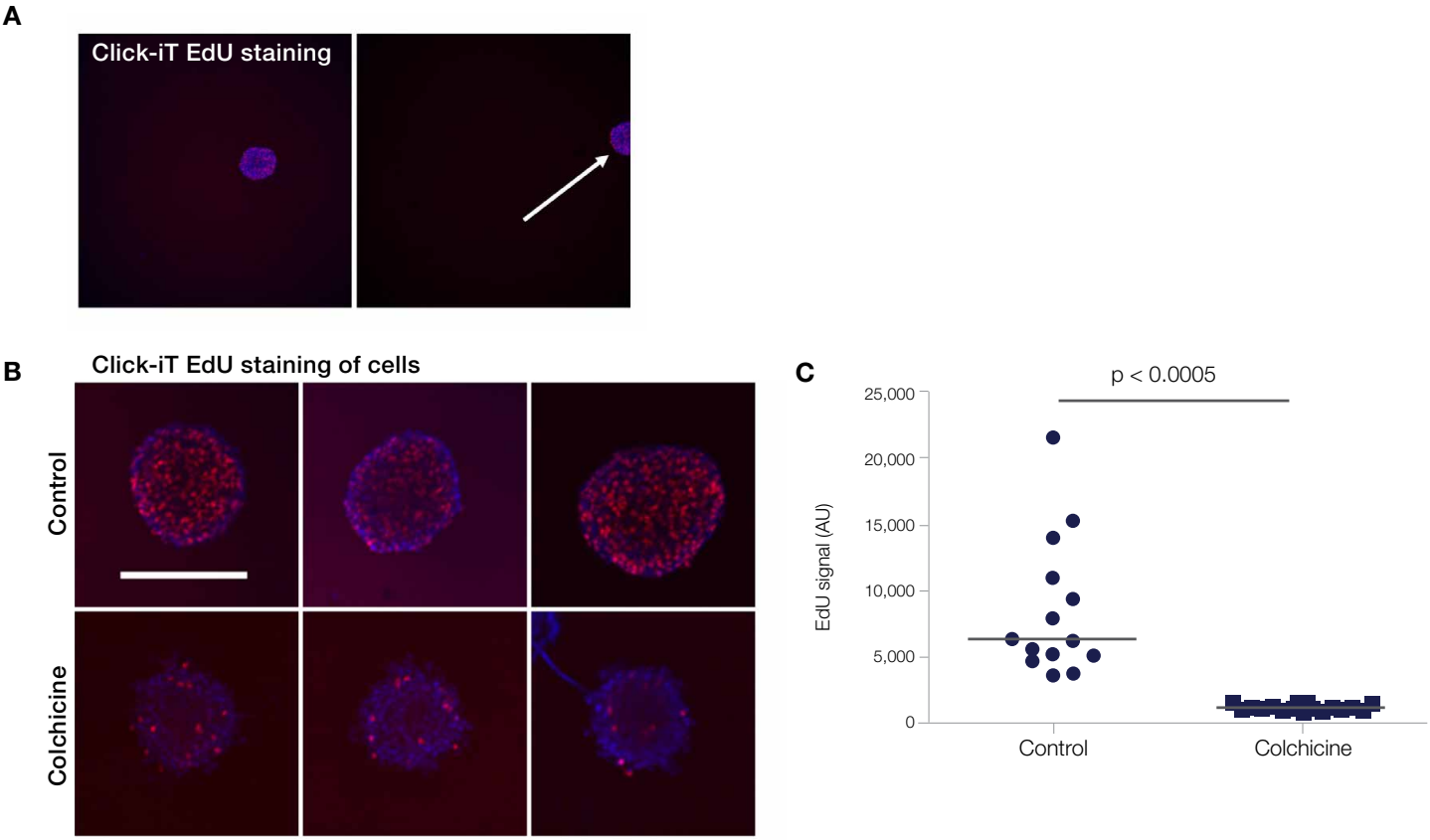

Figure 6. Cell proliferation assay analysis. (A) Field view of a fully captured (left) and a partially captured (right) spheroid as the latter got dislodged during washing. **(B)** Representative images showing Click-iT EdU staining (red) in T-47D spheroids without and with colchicine treatment. Images were acquired using the CellInsight CX7 HCS Platform under a 4x objective and in confocal mode. Scale bar: 200 µm. **(C)** Dot plot analysis of cellular proliferation in T-47D spheroids without and with colchicine treatment. The general intensity measurement tool in HCS Studio software 4.0 was used to analyze the Click-iT EdU signal (y-axis); N = 2. *P* < 0.0005 for difference from control by unpaired *t*-test.

Nevertheless, buffer exchanges should be performed carefully, as scratches in the wells give background signal during imaging, leading to noise in analysis.

For cell proliferation analysis, the spheroid was masked to negate background signal intensity. As depicted qualitatively in Figure 6B and quantitatively in 6C, colchicine treatment led to a significant reduction in proliferating cells in spheroids, indicated by reduced EdU signals.

Conclusion

Though spheroids can be more complicated to analyze than cells cultured under standard 2D conditions, we have shown that a wide variety of cell-based as well as culture supernatant–based assays can be optimized to test drug responses in cancer cells grown in 3D. For the majority of cases, increasing the incubation time of drugs as well as detection reagents for 3D cultures helps reagents better penetrate the spheroids and results in more meaningful data. We recommend keeping washes to a minimum and instead using media exchanges. Based on our observations, centrifuging spheroid-containing plates multiple times does not help to settle spheroids at the bottom, especially if the spheroids are fixed. So, exchanging buffer carefully and gently along the sides of wells is recommended. For comparative studies where analysis can be done on the medium rather than the cells, e.g., PrestoBlue HS reagent or ELISA, a microplate-based readout is the preferred method. However, when the readout is cell based and involves multiple buffer exchange steps, such as the CellEvent Caspase-3/7 Green Detection Reagent for apoptosis studies or Click-iT EdU detection kit for cell proliferation studies, an image-based readout will yield more reliable and reproducible information about the cellular effect of drugs.

Ordering information

Product	Cat. No.
Plastics	
Nunclon Sphera 96-well plates	174925
Nunclon 96-well optical-bottom plates	164588
Matrix Reagent Reservoirs	8094
Media, serum, and antibiotics	
DMEM (PANC-1, MDA-MB-231)	31966021
MEM (HepG2)	11095080
RPMI (LNCaP, T47D)	72400047
McCoy's 5A (SKOV-3)	16600082
Fetal Bovine Serum (FBS)	10270106
Penicillin-Streptomycin	15140122
Reagents and kits	
Phosphate-Buffered Saline (PBS)	10010031
PrestoBlue HS Cell Viability Reagent	P50201
LIVE/DEAD Viability/Cytotoxicity Kit	L3224
NucBlue Live ReadyProbes Reagent	R37605
PSA (Total)/KLK3 Human ELISA Kit	EHKLK3T
CellEvent Caspase-3/7 Green Detection Reagent	C10423
Click-iT Plus EdU Cell Proliferation Kit	C10639
Instruments	
CellInsight CX7 LZR High-Content Screening (HCS) Platform	A46120
Varioskan LUX Multimode Microplate Reader	N16045
EVOS M7000 Imaging System	AMF7000
Pipettes	
Finnpipette F1 Multichannel Pipettes	4661020N, 4661030N

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