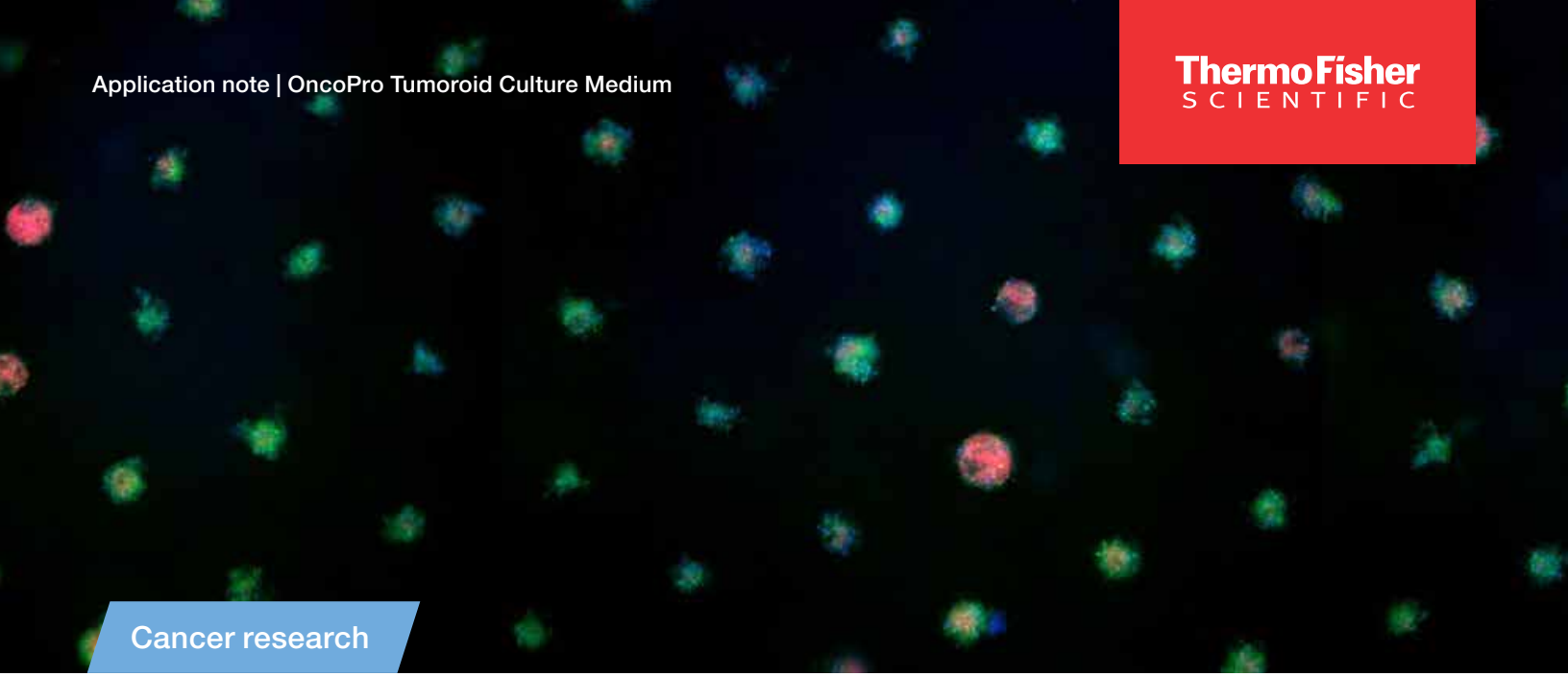


Cancer research

Enabling tumoroid-based compound screens with OncoPro Tumoroid Culture Medium

Keywords
Tumoroids, cancer organoids, patient-derived cancer models, automation, multiplexed readout, high-content imaging

Introduction

Tumoroids, also known as cancer organoids, are patient-derived cancer cells that grow as self-organized 3D multicellular structures. Because tumoroids retain key genetic and transcriptomic features of the originating patient tumors, *in vitro* drug response using these models often correlates with patient clinical outcomes [1]; this also makes tumoroids attractive models for physiologically relevant preclinical compound screens. Despite the promise of tumoroids, researchers have yet to broadly adopt tumoroid technology for their drug screening applications, for several reasons, including:

- Lack of an easy-to-use, standardized tumoroid culture medium
- Limited availability of tumoroid models
- Relative difficulty in scaling up tumoroids to obtain sufficient cells for use in downstream assays

Gibco™ OncoPro™ Tumoroid Culture Medium was developed to expand tumoroid lines with relative ease. The standardized formulation has low lot-to-lot variability, is free of serum and conditioned medium, does not require a Thermo Fisher Scientific license for preclinical commercial use, and is compatible with both embedded and suspension culture methods. Furthermore, the number of components required to prepare the complete medium is reduced compared to homebrew formulations. Here we demonstrate how tumoroids cultured in OncoPro Tumoroid Culture Medium can be readily used in compound screens.

Robust scale-up in suspension culture for downstream assays

Although OncoPro Tumoroid Culture Medium can be used to grow tumoroids in traditional dome culture (embedded in basement membrane extract, or BME), the system is also compatible with a suspension culture method in which tumoroids are free-floating in complete medium supplemented with 2% (v/v) BME in solution. With suspension culture, tumoroids are more easily scaled up to seed a large number of assay plates while retaining gene expression profiles comparable to those grown in embedded culture [2]. To demonstrate scalability and expected cell yield across culture formats, we grew tumoroids in multiple sizes of non-treated flasks (Figure 1). Cell morphology was preserved across formats (Figure 1A, B), and viable cell yield after one week of growth scaled with flask surface area (Figure 1C). Additionally, nearly identical growth rates (assessed as number of population doublings per week) were observed in the different flask formats (Figure 1D). In this case, we recovered over 100 x 10^6 dissociated cells after growth in a Thermo Scientific™ Nunc™ TripleFlask™ Non-treated Cell Culture Flask (Figure 1C). We recommend choosing an appropriate flask size, and a sufficient number of them, to obtain cells for the downstream assay of interest based on the observed doubling times of the tumoroid lines being used. Approximately 1.5 x 10^6 dissociated tumoroid cells are required to seed one full 96-well plate.

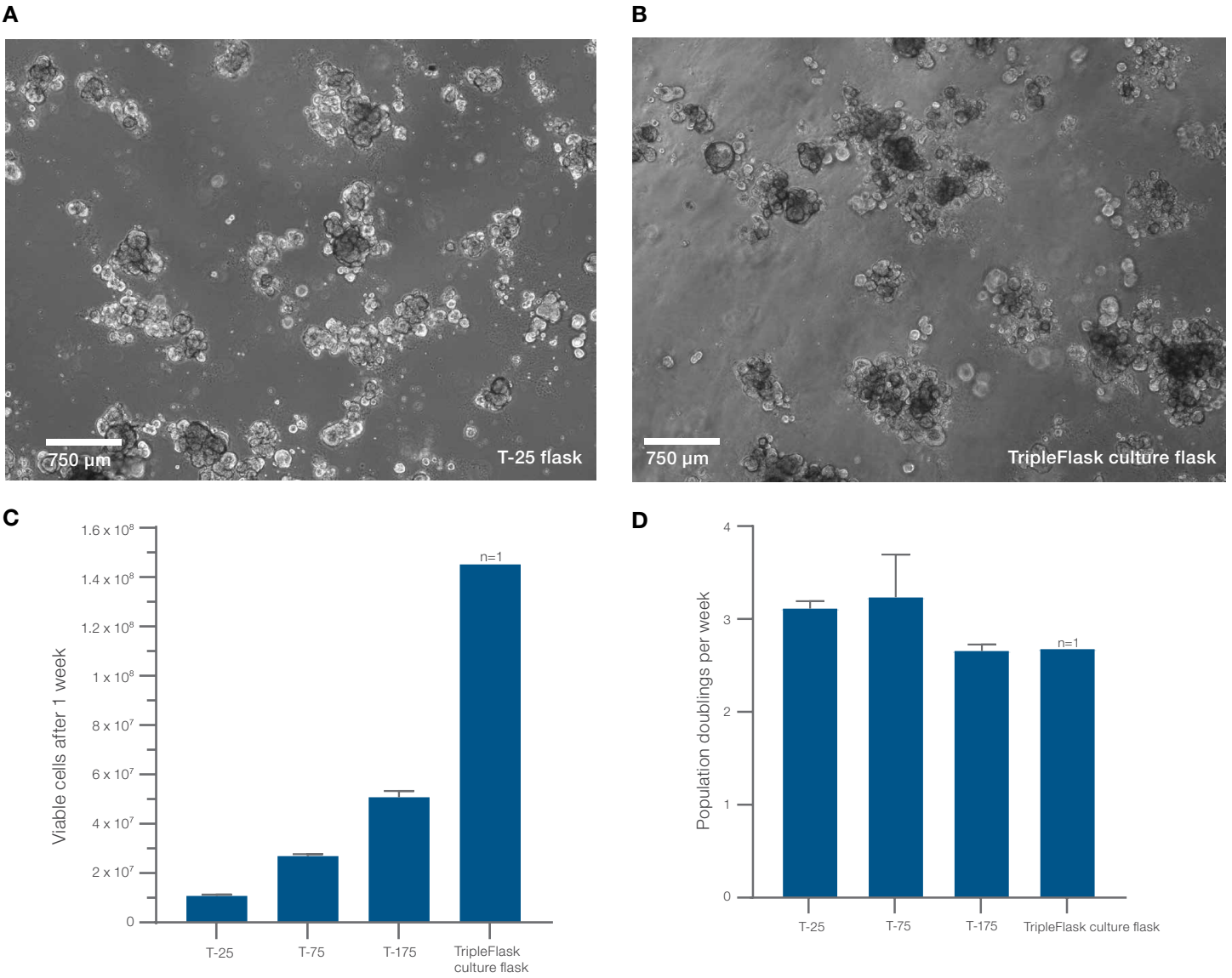


Figure 1. Scale-up of tumoroids in suspension culture for downstream assays. A human colorectal tumoroid model was grown in suspension in multiple non-treated flask formats in OncoPro Tumoroid Culture Medium, and tumoroid morphology and the number of viable cells were compared after one week in culture. Representative images of tumoroids expanded in **(A)** a T-25 flask and **(B)** a Nunc TripleFlask Non-treated Cell Culture Flask are shown. **(C)** Number of cells (mean ± standard deviation) harvested after collection and dissociation from various flask formats following one week in culture. **(D)** Growth rates were quantified as population doublings per week (mean ± standard deviation) and were comparable across flask formats.

Reproducible tumoroid plating using manual or automated methods

Following expansion, tumoroids can be dissociated using the method outlined in the **OncoPro Tumoroid Culture Medium Kit user guide**, counted, and plated for downstream assays. To remove any undissociated clusters of cells and minimize well-to-well variability during plating, we recommend passing cells through a 100 µm mesh cell strainer prior to counting when preparing for compound screens. For most tumoroid lines, this results in minimal loss (<2%) of cells (Figure 2A). After counting, cells should be resuspended in cold OncoPro Tumoroid Culture Medium (supplemented as appropriate for the cancer indication of interest) containing 2–4% (v/v) BME. Published methods indicate that higher concentrations of BME would also be acceptable if desired [3]. An initial titration of BME concentration should be performed to confirm that tumoroid morphology observed when cells are seeded in the scaled-down format for compound screens is comparable to that observed during routine culture [4]. If ROCK inhibition is not expected to affect the mechanism of action of compounds in the screen, we include 10 µM Y-27632 in the resuspension solution. Tumoroids will survive in the absence of Y-27632 but may grow more slowly.

We recommend plating 10,000–15,000 dissociated tumoroid cells in 100 µL per well of a non-treated flat-bottom 96-well plate to enable robust tumoroid formation and consistent seeding. For reference, a resuspension concentration of 150,000 viable cells/mL equates to a plating density of 15,000 cells/well when dissociated tumoroid cells are seeded in 100 µL medium per well in a 96-well plate. This format leads to a coefficient of variation (CV) of less than 10% between wells, measured one day post-seeding using Invitrogen™ PrestoBlue™ HS Cell Viability Reagent in a 96-well plate (Figure 2B).

Other seeding formats can also be utilized. Microcavity plates, for example, promote the formation of uniform tumoroids by physically segregating dissociated cells into multiple discrete recesses per microplate well prior to tumoroid growth. Dissociated tumoroid cells can be seeded in microcavity plates at the cell densities specified above to generate tumoroids for testing. Such formats can lead to strikingly uniform distributions of tumoroid number (per 96-well plate well) and size across wells up to 7 days post-seeding (Figure 2C–F).

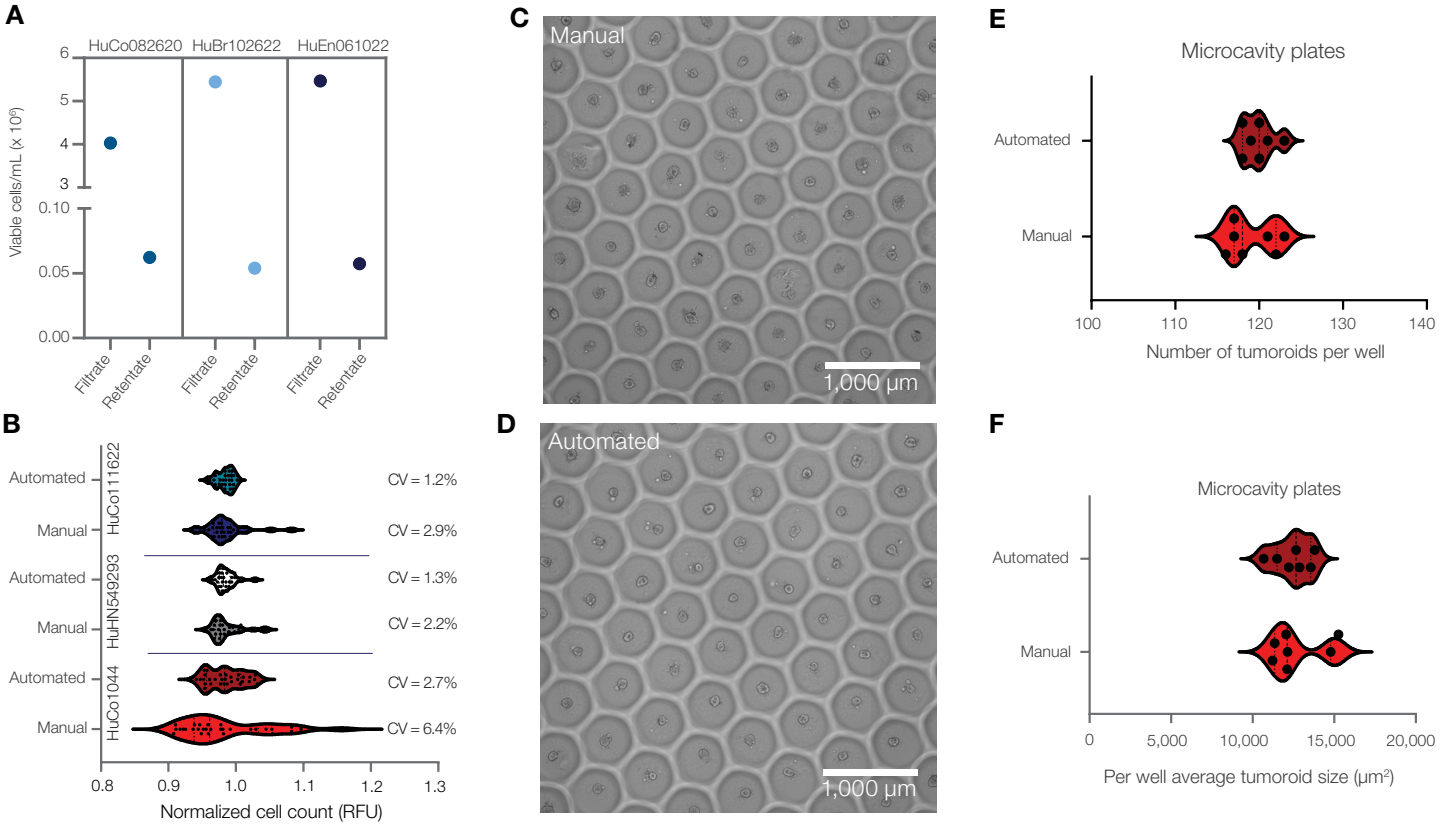


Figure 2. Handling and plating of dissociated tumoroids for compound screening assays. Various tumoroid models were expanded in suspension culture, dissociated, and plated. Plating was performed using manual and automated methods. **(A)** Cell counts of filtrate and retentate following straining of dissociated cells through a 100 µm mesh. **(B)** Well-to-well variability in cell count at one day post-plating (measured using PrestoBlue HS Cell Viability Reagent, relative fluorescence units (RFU) normalized to average value for a given condition). Each point represents one well of a flat-bottom 96-well plate, with 30 wells per condition. **(C, D)** Images of colorectal cancer tumoroids formed in microcavity plates 7 days after seeding by manual and automated methods. **(E)** Number of tumoroids per well and **(F)** average tumoroid area were well controlled in microcavity plates. Each point represents one well, with 7 wells analyzed per condition.

Dissociated tumoroid cells resuspended in OncoPro Tumoroid Culture Medium are also compatible with automated fluid handling, which can further minimize well-to-well variation (Figure 2B–F). Due to the propensity of tumoroids to settle out of suspension, triturating a volume identical to the seeding volume 3–5 times prior to drawing up the cell suspension is critical for uniform seeding when using automated liquid handlers; the speed of aspiration did not have a significant impact on seeding variability in our studies. The vessel in which cells are placed prior to seeding also impacts variability. For our purposes, with a cell suspension volume of about 20 mL, a 50 mL trough provided the best results and minimized seeding variation. Larger troughs led to uneven cell distribution when performing assays with smaller volumes of the cell suspension.

Multiplexed readouts for plate reader–based analysis

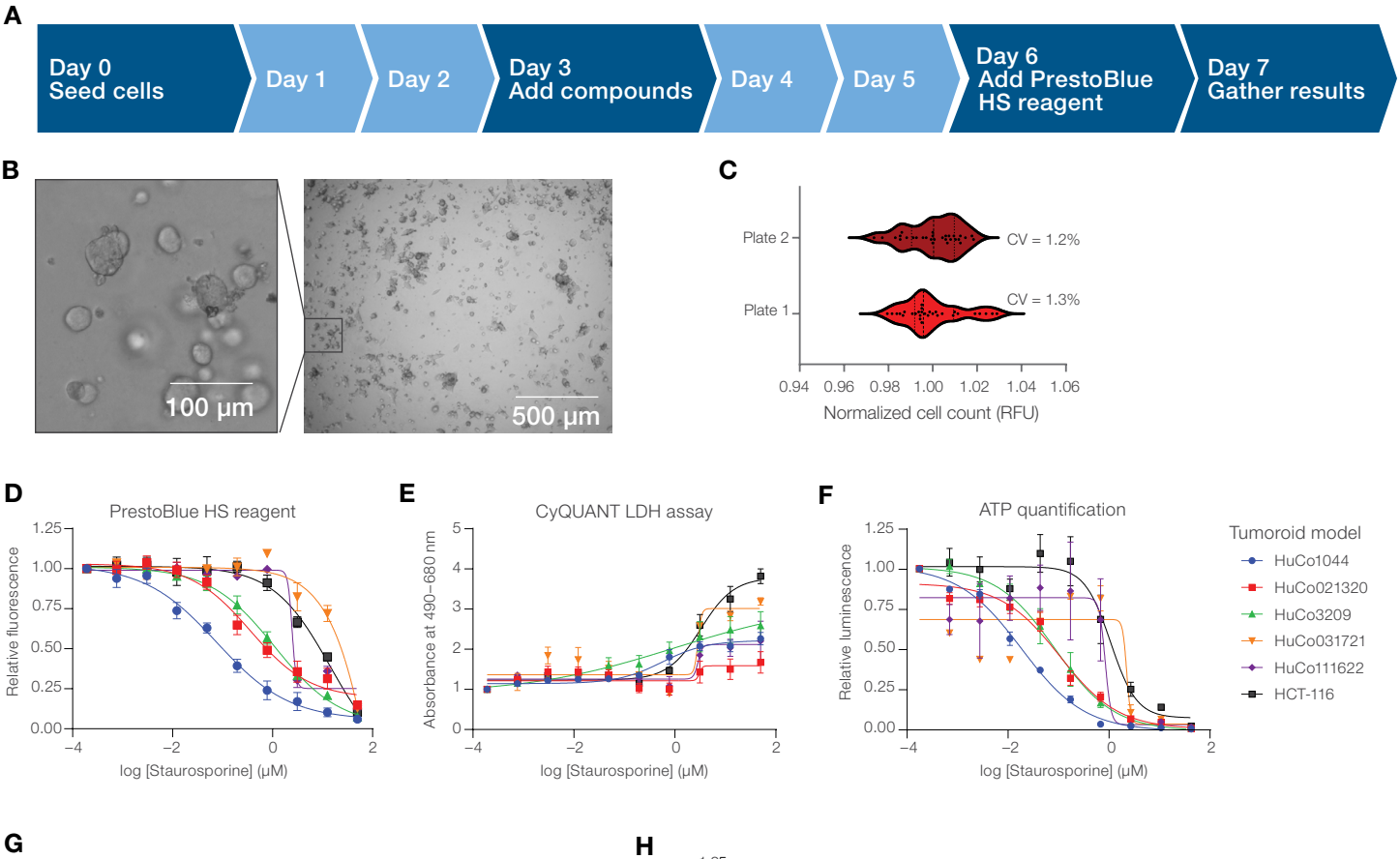
To allow for tumoroid formation following plating, we typically maintain cultures for 72 hr in OncoPro Tumoroid Culture Medium prior to the addition of compounds (Figure 3A). For 96-well flat-bottom plates, following the cell seeding recommendations specified above led to a range of tumoroid sizes but consistent well-to-well seeding (Figure 3B, C). At the time of screening initiation, compounds can be added at 2X concentration in 100 µL of OncoPro medium containing BME, to reach a final volume of 200 µL per well in 96-well plates, with a 1X final drug concentration in each well. Appropriate positive and negative controls should be included in all screening assays.

To demonstrate the use of tumoroids expanded in OncoPro Tumoroid Culture Medium in compound screens, we performed proof-of-concept experiments using 9-point staurosporine dose-response curves (plus a negative control) across 5 colorectal tumoroid lines, with response measured across triplicate wells for a given staurosporine concentration for each biological run. An immortalized colorectal cancer cell line, HCT-116, was grown in 3D in OncoPro Tumoroid Culture Medium and tested in parallel for comparison. Experiments were repeated to obtain 2–3 biological replicates. We used an initial seeding density of 15,000 tumoroid cells per well in OncoPro Tumoroid Culture Medium supplemented with 4% BME. This method is compatible with standard plate reader–based viability assays, including the PrestoBlue HS viability assay, Invitrogen™ CyQUANT™ LDH Cytotoxicity Assay, and ATP quantification. In these experiments, PrestoBlue HS reagent was added to each well (22 µL/well, for a final concentration of 10%) on day 6 and read after a ~16 hr incubation on day 7 using the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader in bottom-read fluorescence mode (Figure 3D). For 3D tumoroid models, we have found that this longer incubation with PrestoBlue HS reagent is critical for the signal to stabilize and generate accurate results. After taking readings of the PrestoBlue HS reagent, 50 µL of cell-free supernatant was sampled and analyzed for lactate dehydrogenase content using the CyQUANT LDH assay (Figure 3E). Cells remaining in the plate were used for luminescence-based ATP quantification as a third measure of cell viability (Figure 3F). Colorectal tumoroid lines derived in OncoPro Tumoroid Culture Medium showed a wide range of response to staurosporine and were generally more sensitive to this compound than HCT-116 cells, though one line (HuCo031721) displayed marked staurosporine resistance. The rank orders of IC_{50} values were nearly identical for the PrestoBlue HS assay and ATP quantification, though users should be aware that the contrasting mechanisms of action of the viability assays can generate differences in the absolute values of the IC_{50} results calculated from a four-parameter variable slope least squares fit (Figure 3G).

Product highlights

- **PrestoBlue HS Cell Viability Reagent**—No-wash, resazurin-based indicator of cell viability that is compatible with live-cell analysis. PrestoBlue HS reagent contains resazurin, a compound that is reduced to resorufin (red fluorescence) in living cells. Therefore, the PrestoBlue HS reagent signal (measured by fluorescence- or absorbance-based instrumentation) is lower in conditions in which fewer viable cells are present. The high signal-to-background ratio of PrestoBlue HS reagent generates robust data from 3D culture models. As this reagent is nontoxic, cells analyzed with PrestoBlue HS reagent can be further cultured, lysed for analysis using other viability assays, or fixed for downstream imaging.
- **CyQUANT LDH Cytotoxicity Assay**—Add-mix-read reagent to detect lactate dehydrogenase (LDH), a cytosolic enzyme that is released upon plasma membrane damage, in cell culture supernatant as a measure of cytotoxicity. The CyQUANT LDH reagent signal (measured by absorbance-based instrumentation) increases with increasing cell death. As this assay measures extracellular LDH, samples can be monitored over time by sequential sampling of cell culture supernatants, and cells remaining in culture can be used for further analysis.
- **Varioskan LUX Multimode Microplate Reader**—Versatile microplate reader that can be configured for top and bottom reading of absorbance, fluorescence, luminescence, and time-resolved fluorescence measurements. Here the Varioskan LUX microplate reader was used to measure cell viability using indicators based on fluorescence (PrestoBlue HS reagent), absorbance (CyQUANT LDH reagent), and luminescence (ATP quantification).

We have achieved comparable screening results when plating cells, adding compounds, and adding detection reagents manually or with the use of an automated liquid handler (Figure 3H). In this example, tumoroids were treated with either staurosporine or azaserine, and similar IC_{50} values were obtained using either manual or automated liquid handling. If using liquid handlers to remove supernatant for LDH analysis or prior to the addition of lysis buffer for ATP quantification, we recommend using liquid detection features (when possible) instead of setting a static aspiration height. Liquid detection allows for consistent aspiration of fluid from wells and minimizes variability that may be due to evaporation during incubation periods. A distance of 2 mm below the liquid surface has worked well in our hands when using flat-bottom 96-well plates. Optimizing this setting enables consistent removal of a defined volume of medium while retaining cells in the original plate.



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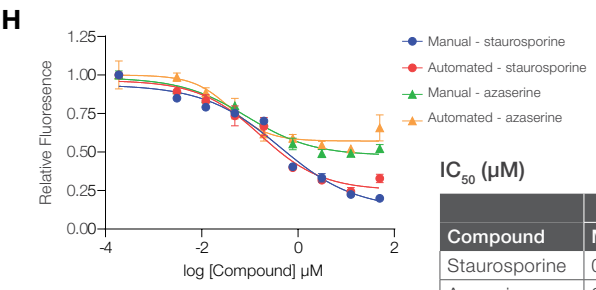
Staurosporine IC_{50} (µM)

	Cell viability reagent		
Tumoroid model	PrestoBlue HS	ATP quantification	CyQUANT LDH
HuCo1044	0.07657	0.0207	0.564
HuCo021320	0.3631	0.12	3.053
HuCo3209	1.102	0.0967	0.993
HuCo111622	2.453	0.963	2.88
HuCo031721	273.1	2.584	2.87
HCT-116	14.42	1.23	3.27



IC_{50} (µM)

	Handling method	
Compound	Manual	Automated
Staurosporine	0.418	0.167
Azaserine	0.0864	0.0392

Figure 3. Compound screening using colorectal tumoroid lines. (A) Overview of tumoroid compound screening assays. PrestoBlue HS Cell Viability Reagent was added on day 6 and the signal read on day 7. **(B)** Representative images of tumoroids at the time of compound addition (day 3). **(C)** Well-to-well variability in normalized fluorescent readings using PrestoBlue HS Cell Viability Reagent on day 3 following seeding in a flat-bottom 96-well plate. Plots display distribution of per-plate normalized values across two plates, with each point representing one well (N = 30 wells per plate). **(D–F)** Dose-response curves for colorectal tumoroid lines and HCT-116 colorectal cancer cells treated with staurosporine and assayed using PrestoBlue HS Cell Viability Reagent, CyQUANT LDH Cytotoxicity Assay, and luminescence-based ATP quantification. Values were normalized to a negative control prior to curve fitting, and mean values ± standard error of the mean (SEM) across 2–3 biological replicates are displayed. **(G)** IC_{50} values for staurosporine treatment of colorectal tumoroid models and HCT-116 cells (grown in 3D). Table entries are color-coded according to IC_{50} values obtained with a given cell viability reagent (higher values in red, lower values in blue). **(H)** Dose-response curves for HuCo111622 colorectal tumoroids treated with staurosporine or azaserine, where fluid handling was either manual or with an automated liquid handler. Mean values ± SEM of PrestoBlue HS reagent fluorescence (normalized to negative control) across 2–3 technical replicates per condition are displayed. IC_{50} values are shown in the table.

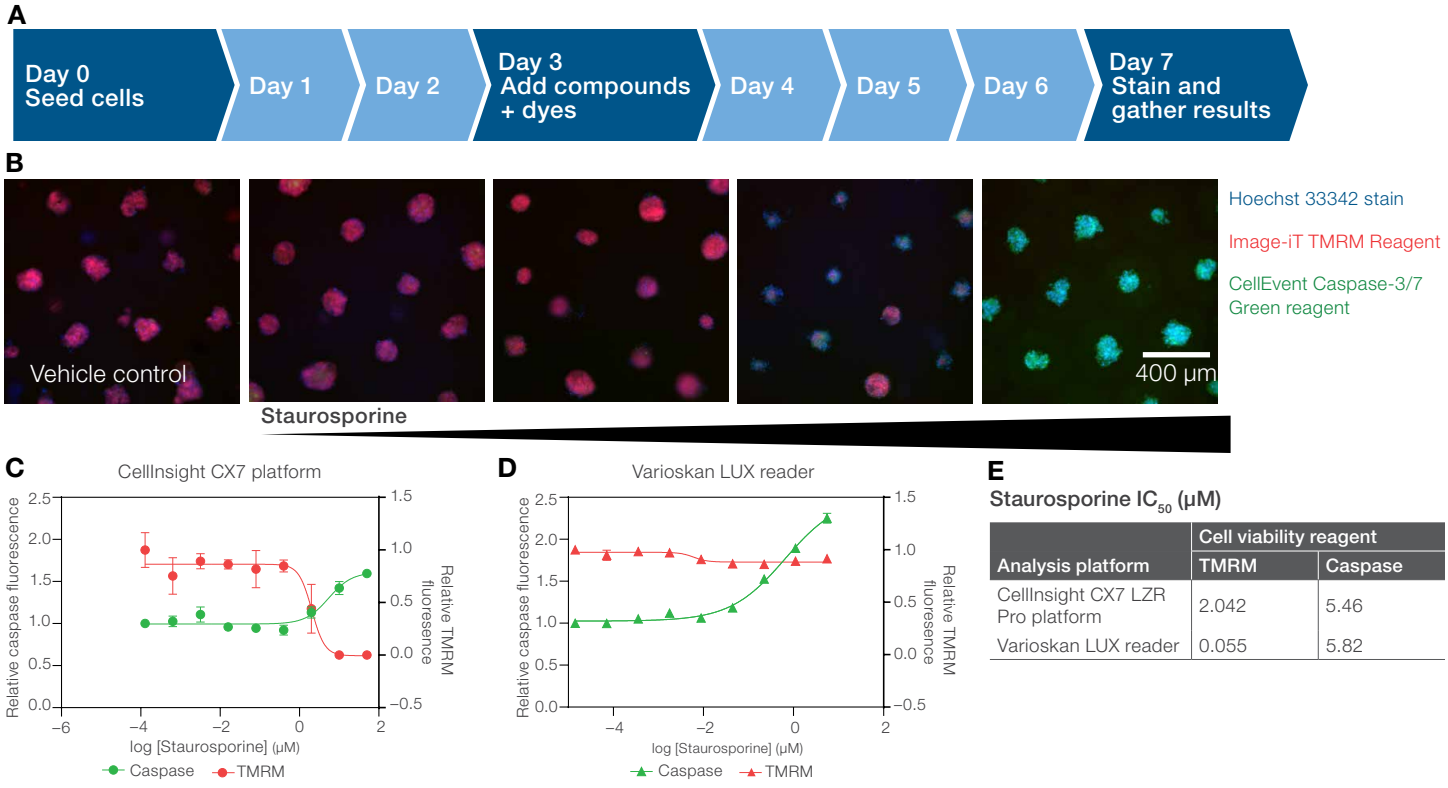
Assay tips

- Keep cell suspensions on ice prior to addition of BME, to avoid polymerization, which could result in uneven aggregation and cell seeding.
- Incubate tumoroids with PrestoBlue HS reagent overnight (~16–24 hr) to obtain reliable results. For 3D models, shorter incubation times will not yield steady-state fluorescence values from PrestoBlue HS reagent.
- Incubation times with fluorescent dyes are typically longer for 3D tumoroids than for 2D cell lines. Live cell–compatible dyes such as Invitrogen™ Image-iT™ TMRM Reagent and Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent can be included in OncoPro Tumoroid Culture Medium for real-time analysis of cell health.

Compatibility with high-content imaging

Imaging of tumoroids following the application of pharmacologically active compounds may be beneficial in some instances due to the morphological complexity of 3D models. To demonstrate the compatibility of tumoroids with high-content imaging approaches and assay results on a tumoroid-by-tumoroid instead of well-by-well basis, we performed a proof-of-concept compound screen using fluorescent probes for cell health as our primary readouts. All cell seeding and fluid transfer steps were performed with an automated liquid handler.

Tumoroids were plated in microcavity plates, and compounds were added on day 3 in the presence of 133 nM Image-iT TMRM Reagent and 10 µM CellEvent Caspase-3/7 Green Detection Reagent (Figure 4A). On day 7, fresh medium containing detection reagents and 2.5 µg/mL Invitrogen™ Hoechst™ 33342 stain was added 1–2 hr prior to imaging on the Thermo Scientific™ CellInsight™ CX7 LZR High Content Screening Platform. Tiled images of each well were collected with a 4x objective (Figure 4B) and analyzed using the Spot Detector tool, with each tumoroid gated as one spot using the Hoechst 33342 dye signal. For each well, the total fluorescence intensity per tumoroid (green channel, CellEvent Caspase-3/7 Green Detection Reagent) and average fluorescence intensity per tumoroid (red channel, Image-iT TMRM Reagent) were calculated, normalized to negative control values, and plotted to obtain dose-response curves (Figure 4C). The same plate was also read using the Varioskan LUX Multimode Microplate Reader in bottom-read fluorescence mode (Figure 4D). Analysis on the CellInsight CX7 LZR platform provided markedly improved dynamic response, particularly for the Image-iT TMRM Reagent, and a clear crossover concentration at which cell death occurred. Dose-response curves using CellEvent Caspase-3/7 Green reagent were similar using both analysis platforms. The staurosporine IC_{50} values calculated from data generated using each platform are summarized in Figure 4E.



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Staurosporine IC_{50} (µM)

Analysis platform	Cell viability reagent	
	TMRM	Caspase
CellInsight CX7 LZR Pro platform	2.042	5.46
Varioskan LUX reader	0.055	5.82

Figure 4. Image-based analysis of cell viability during compound screens. (A) HuCo1044 colorectal tumoroids were dissociated, seeded in microcavity plates, and exposed to staurosporine and cell viability dyes after 3 days. Fresh medium containing detection reagents and Hoechst 33342 stain was added on day 7, and tumoroids were imaged. **(B)** Representative images of tumoroids at increasing staurosporine doses. Images were acquired on the CellInsight CX7 LZR platform. **(C, D)** Dose-response curves for staurosporine treatment of HuCo1044 tumoroids following analysis on the CellInsight CX7 LZR platform or Varioskan LUX microplate reader. Data show mean ± SEM across three wells per condition. **(E)** IC_{50} values for staurosporine treatment of HuCo1044 tumoroids, for each analysis platform and cell viability reagent.

Product highlights

- **CellEvent Caspase-3/7 Detection Reagents**—Add-and-read, low-background fluorogenic detectors of caspase-3/7 activity in cells undergoing apoptosis, available in both green- and red-fluorescent versions. CellEvent Caspase-3/7 Detection Reagents are compatible with live-cell imaging and with formaldehyde-based fixation. Here, CellEvent Caspase-3/7 Green Detection Reagent was used to measure apoptosis in response to increasing concentrations of staurosporine.
- **Image-iT TMRM Reagent**—Cell-permeant dye that accumulates in functional mitochondria of live cells with healthy membrane potentials. As cells die and membrane potential is lost, the TMRM signal will dim or disappear. Image-iT TMRM Reagent is not fixable and is therefore suitable for live-cell analysis.
- **CellInsight CX7 LZR Pro High Content Screening Platform**—High-content analysis platform for acquisition and quantification of cell-based assay data. Laser-based illumination and integrated spinning-disc confocal technology make the CellInsight CX7 LZR Pro platform a versatile instrument for high-content analysis of 3D cell culture models.

Conclusion

Tumoroid models are increasingly being used in preclinical compound screens to generate unique biological insights, because of their well-aligned representation of patient tumors. OncoPro Tumoroid Culture Medium enables consistent growth of tumoroid models using a standardized base medium. Careful handling of dissociated tumoroid cells using either manual or automated methods enables even cell seeding across plate wells. Additionally, the flexibility to grow large numbers of tumoroid cells in suspension culture facilitates downstream assays. Similar scale-up and scale-down methods have been applied to use tumoroids in immune cell cytotoxicity assays [5]. A number of the tumoroid lines presented here are available for purchase through our **Cell Biology Services** to facilitate use of tumoroids in applications of interest. Tumoroid models will serve as a valuable and physiologically relevant resource to accelerate drug discovery and development.

Acknowledgments

NCI PDMR model 549293-155-R-V1-organoid (abbreviated HuHN549293), received from the PDMR at passage 18, was used to generate data presented in Figure 2B. We thank the NCI PDMR and their contributing institutions for their contributions to this work.

Ordering information

Product	Cat. No.
OncoPro Tumoroid Culture Medium Kit	A5701201
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	A1413201
CellEvent Caspase-3/7 Detection Reagents	C10423
Heat Stable FGF-10 Recombinant Protein	PHG0371
PrestoBlue HS Cell Viability Reagent	P50201
CyQUANT LDH Cytotoxicity Assay	C20300
Image-iT TMRM Reagent (mitochondrial membrane potential indicator)	I34361
Hoechst 33342, Trihydrochloride, Trihydrate	H3570
Varioskan LUX Multimode Microplate Reader	VLBL0TGD1
CellInsight CX7 LZR Pro High Content Screening Platform	HCSDCX7LZRPRO

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