SPHEROIDS

rapidly becoming a preferred 3D culture format

There are many advantages associated with culturing cells in three-dimensional (3D) versus conventional two-dimensional (2D) tissue culture. Scaffold-free 3D culture systems that generate spheroids (and other similar multicellular aggregations) have proved useful as they offer an easy route to access 3D culture and transition into plate-based higher throughput. The emergence of ultra-low attachment (ULA) or cell-repellent plates that generate spheroids has been a major factor in increasing investigation and adoption of 3D culture. The popularity of ULA plates for spheroid generation has shifted the emphasis in spheroid generation to size and shape consistency, defined co-cultures with primary cells and optimising conditions for those 'difficult to grow' cells. Much progress has been made in recent years in the supporting literature, culture tools, detection technologies, reagents and service offerings enabling spheroids to be adopted as the preferred *in vitro* 3D platform in safety testing, disease modelling and drug discovery applications. Spheroids are also proving invaluable in regenerative medicine/tissue engineering research where they are used as a biological unit or building block to merge into a more complex tissue or organ structures.

By Dr John Comley

Interest in three-dimensional (3D) cell culture in drug discovery has intensified in recent years as the potential advantages of such systems over conventional two-dimensional (2D) tissue culture become evident. The benefits include the ability of 3D to provide a more physiologically-relevant environment, organ-like microarchitecture, morphology more predictive of disease states and *in vivo*-like drug responses. One type of 3D structure generated *in vitro* using cell culture systems is spheroids. These in their various forms may also be referred to as microtissues, embryoid bodies, organoids or tumourspheres and typically are spherical aggregates of proliferating, quiescent and necrotic cells that in culture retain 3D architecture and tissue-specific functions. In recent years 3D spheroid culture systems have come to the fore in the areas of cancer research, drug discovery and toxicology. Some of reasons why interest in spheroids has taken off include the similarity to the way tumours develop and therefore have relevance to studying tumour biology; they are amenable to

Figure 1: Most important criteria for choosing to focus on 3D spheroids

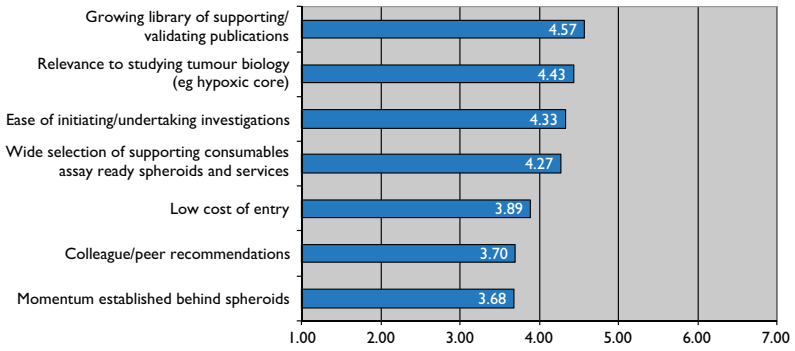


Figure 2: Main area of interest/activity involving 3D spheroids

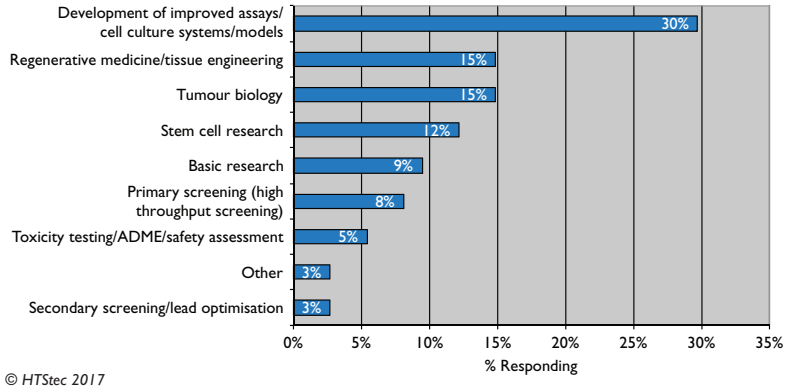
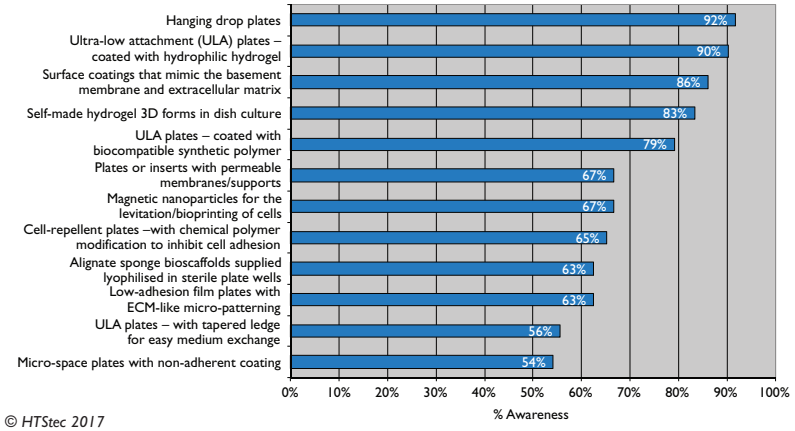


Figure 3: Awareness of approaches currently available to generate/culture 3D spheroids



generation in large numbers of relatively consistent size and shape; and they can be counted, morphologically measured and analysed by a variety of techniques and instruments. This has spawned significant vendor innovation in spheroid-generating consumables as well as application notes on how to undertake their image analysis. Some of the initial research on spheroids focused on their formation in hanging drop cultures, although the automation of spheroid creation and handling in hang drops plates has hindered the widespread adoption of this approach. Subsequently, a range of alternative microplate-based methods have emerged which are simpler to initiate, offer low cost of entry and are amenable to higher throughput and screening. The most successful of these is the ultra-low attachment (ULA) or cell-repellant plate. A variety of chemistries (surface coatings) are used to generate the ultra-low cell attachment, but the effect is basically the same, ie cell immobilisation is inhibited, cells are forced into a suspended state and they clump together enabling 3D spheroid formation. ULA plates have contributed to establishing a momentum behind spheroids that has fostered additional developments and a growing library of supporting/validating publications.

In October 2016, HTStec undertook a market survey on 3D spheroid culture technology[1]. The main objectives of the survey were to understand respondents' current interest in, experience of and progress made in applying 3D spheroid culture/generation technologies. In this article highlights from the market survey are reported and the findings are discussed together with vendor updates on their latest 3D spheroid culture technology.

Why have spheroids gained traction?

Survey respondents ranked growing library of supporting/validating publications as the most important criteria for choosing to focus research on 3D spheroids versus other approaches to 3D culture. This was followed by relevance to studying tumour biology (eg hypoxic core); ease of initiating/undertaking investigations; and wide selection of supporting consumables, assay ready spheroids and services. Ranked least important was the momentum established behind spheroids (**Figure 1**).

Main interest/activity involving 3D spheroids

The main area of interest/activity involving 3D spheroids to survey respondents was development of improved assays/cell culture systems/models (30%). This was followed by regenerative medicine/tissue engineering (15%); tumour biology

(15%); stem cell research (12%); basic research (9%); primary screening (high throughput screening) (8%); toxicity testing/ADME/safety assessment (5%); other (3%); and then secondary screening/lead optimisation (3%) (**Figure 2**).

Awareness and use of technologies

The approaches/technologies survey respondents had greatest awareness of today (ie most heard about and/or most used) to generate/culture 3D spheroids was hanging drop plates (92% aware). This was closely followed by ultra-low attachment (ULA) plates – coated with hydrophilic hydrogel (90%); surface coatings that mimic the basement membrane and extracellular matrix (86%); self-made hydrogel 3D forms in dish culture (83%); and then ULA plates – coated with biocompatible synthetic polymer (79%). Respondents were least aware of micro-space plates with non-adherent coating (54%) (**Figure 3**).

The commercial technologies/products for 3D cell culture/generation/assay most used by survey respondents were: Corning® Costar® ULA hydrophilic hydrogel coated plates (41% using); Greiner Bio-One CELLSTAR® Cell-Repellent Surface plates (14%) and InSphero's GravityPLUS™ Hanging Drop System or InSphero's Gravity TRAP™ ULA Plate with tapered ledge (both 12%). Least used for 3D cell culture/generation/assay was NOF America/Amsbio Lipidure®-COAT plates with biocompatible synthetic polymer film (no respondents using) (**Figure 4**).

Preferred 3D spheroid culture format

The kind of 3D spheroid culture format survey respondents most want to achieve used a round (U)-bottomed 96-well microplate, had a single spheroid per well or drop, and the spheroids were 100-200um diameter. With a significant proportion (42%) of survey respondents indicating they have a specific requirement to transfer spheroids out of their original culture vessel. The type of spheroid culture survey respondents are most interested in investigating was both proliferative and quiescent spheroid culture (56%). This was followed by proliferative spheroid culture, with media exchange, not metabolically stressed, high glucose (37%) and then quiescent spheroid culture, no media exchange, nutrient deprived, low glucose (6%) (**Figure 5**). The cell types most used by survey respondents to generate 3D spheroids today were tumour cell lines (38% of all spheroids). This was followed by primary cells (19%) and then iPSC derived cells (14%). All other cell types had 10% or less use (**Figure 6**). Survey

Figure 4: Direct use of the some commercial technologies/products promoted for 3D spheroid/culture/generation/assay

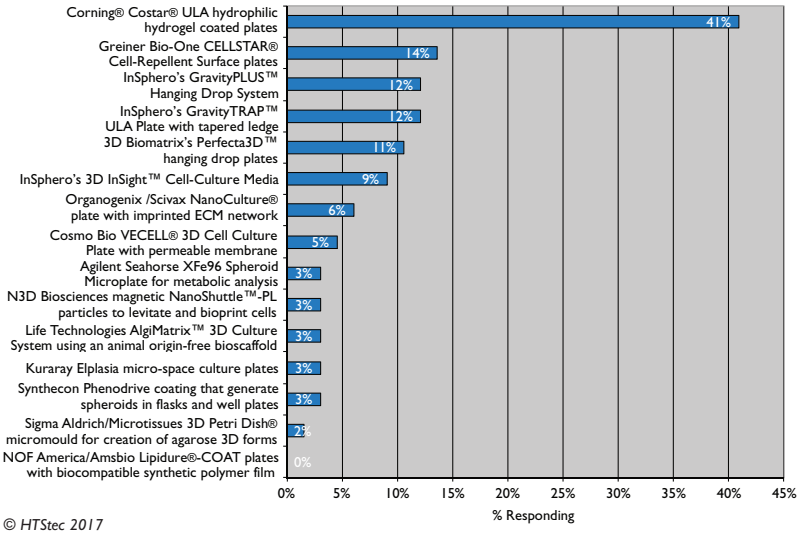


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Figure 5: Type of spheroid culture respondents are most interested in investigating

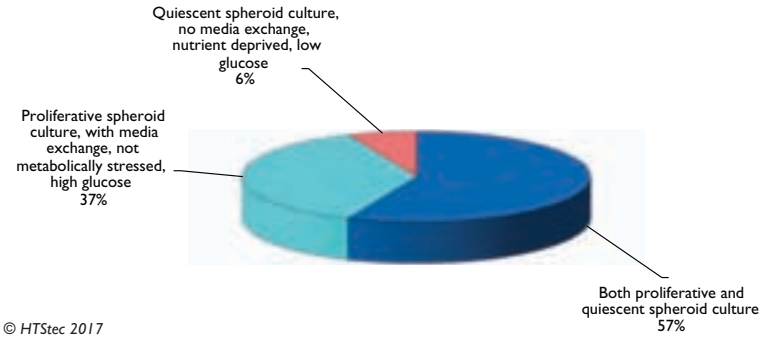

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Figure 6: Different cell types used to generate 3D spheroid cultures today

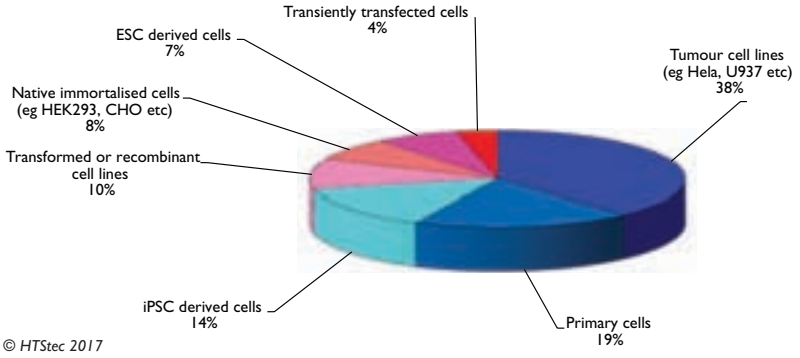

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Figure 7: Number of different cell types per spheroid generated

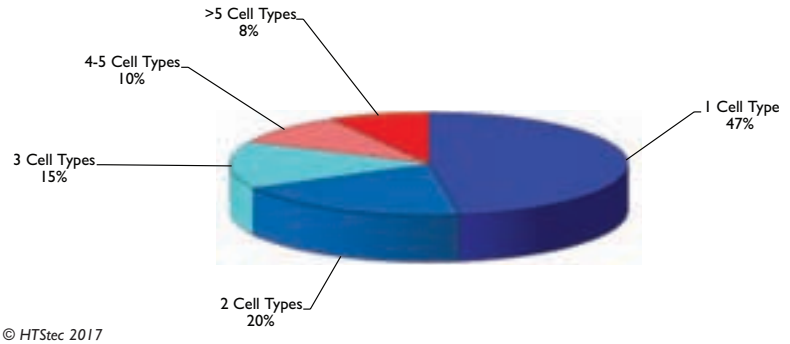


Figure 8: Expectations for primary screening using 3D spheroids vs conventional 2D tissue culture

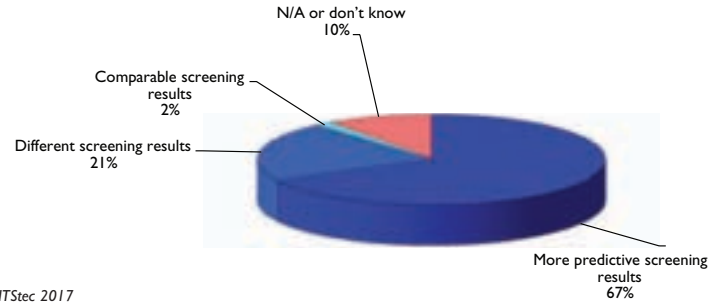
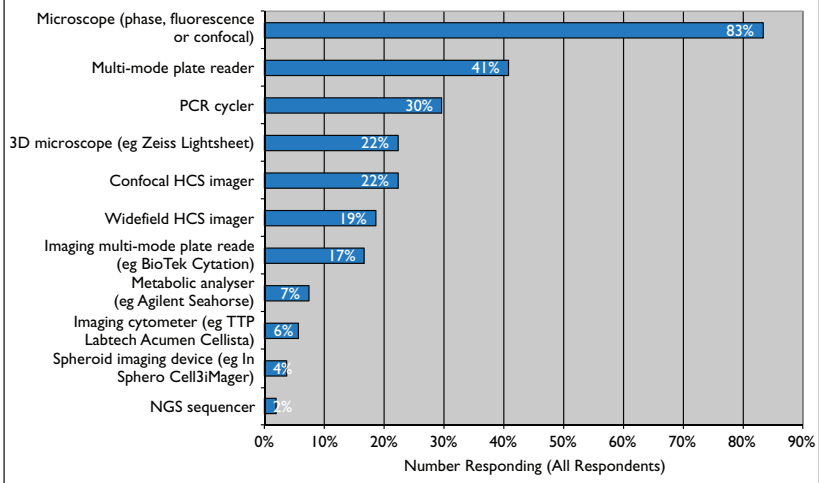


Figure 10: Detection instruments frequently used to assess/monitor spheroid cultures



respondents reported a median of two different cell types per spheroid generated today (**Figure 7**).

Expectations for primary screening

Survey respondents' expectations from primary screening using 3D spheroids versus conventional 2D tissue culture was more predictive screening results (67%). This was followed by different screening results (21%); N/A or do not know (10%); and comparable screening results (2%) (**Figure 8**).

Assay types and detection instruments used to assess/ monitor spheroid cultures

The assay types survey respondents have most applied or plan to apply to isolated spheroids was cell viability (eg luciferase or ATP assay) (75%). This was followed by cell proliferation assay (eg GFP fluorescence) (63%); cell differentiation (55%) and then high content screening/live cell imaging (47%). Least interest in applying was electrical impedance spectroscopy and acoustic/photoacoustic imaging (**Figure 9**). The microscope (phase, fluorescence or confocal) was the detection instrument most frequently used by survey respondents to assess/monitor spheroid cultures. This was followed by the multi-mode plate reader and then PCR cycler. Least used was the NGS sequencer and a specific spheroid imaging device (**Figure 10**). Survey respondents rated cell viability (live/dead) assessment as what they most wanted to achieve from monitoring/assessing spheroid cultures. This was followed by size measurements (perimeter/ diameter/estimated volume/sphericity etc); cell proliferation; and then detailed histology/cellular morphology. All respondents least want to achieve protein interaction (FRET, BRET) from assessing spheroid cultures (**Figure 11**).

Leading players in spheroid generation/culture

The vendor/supplier that first comes to mind as the leading player in spheroid generation/culture was Corning (38% of all vendor selections). This was followed by InSphero (22%); 3D Biomatrix (17%); Thermo Fisher Scientific (12%); Kuraray (2%); Griener Bio-One (2%); Sigma Aldrich (5%); and All others (5%) (**Figure 12**). It should be stated here that this is not a market share projection but an indication of brand awareness.

Figure 9: Assay types have applied or plan to apply to isolated spheroids

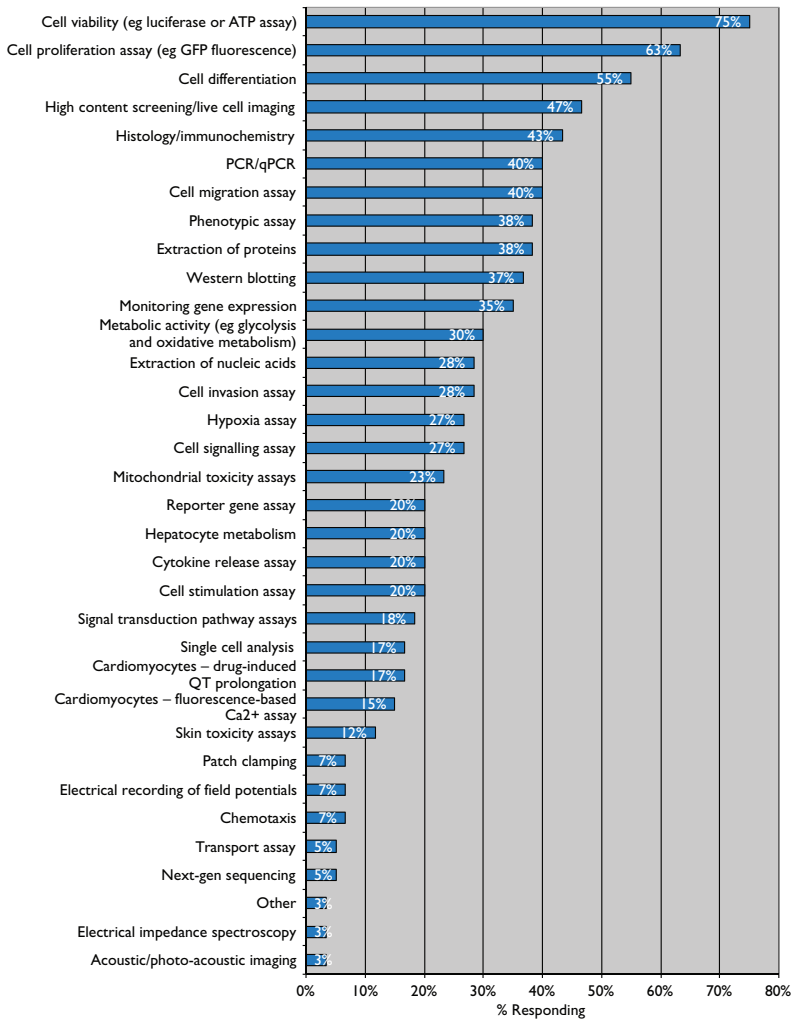


Figure 11. What Respondents Want To Achieve By Monitoring/Assessing Spheroid Cultures

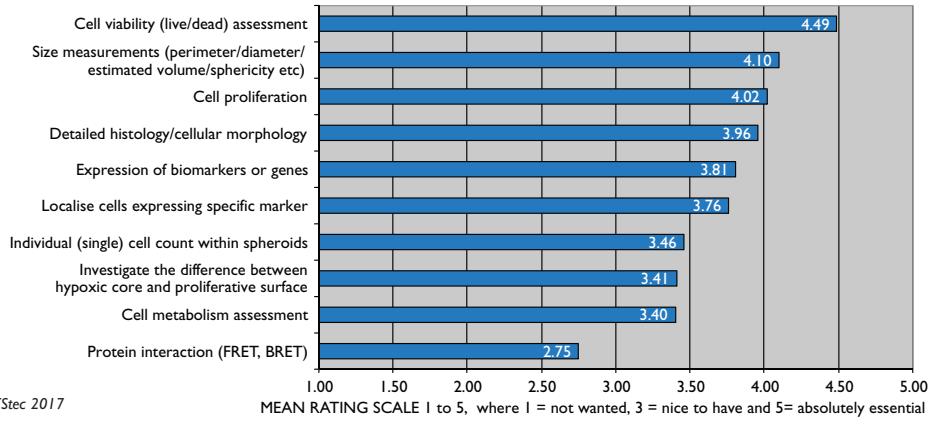
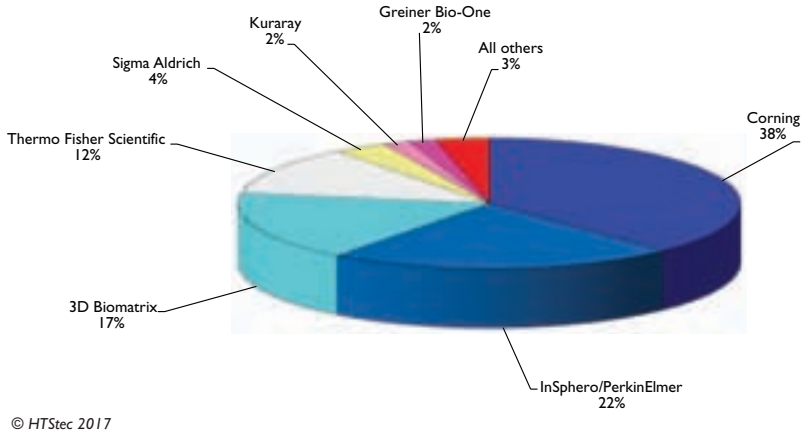


Figure12: Vendor/supplier that first comes to mind as the leading player in spheroid generation/culture



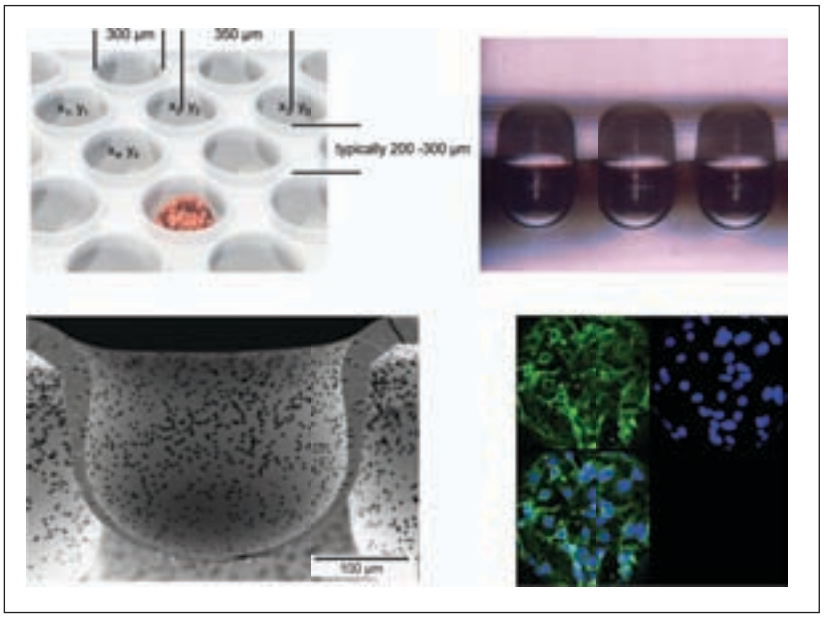


Figure 13: 300MICRONS microcavity arrays with principal design characteristics (upper left), light microscopic image (upper right), after the introduction of pores (lower left, scanning electron microscopy image) and appearance in high-throughput-/high-content-screening applications (lower right, confocal laser scanning microcopy image)

Latest developments in 3D spheroid culture technology

The following vendor snapshots describe some of the latest developments in 3D spheroid culture technology:

By using thin polymer-films (7-10µm) integrated into standard (microtiter plates, multiwell dishes, inserts, etc) or custom formats, **300MICRONS** (www.300microns.com) has developed a new 3D cell culture platform: microcavity arrays. Typically 300µm in diameter and up to 300µm deep, the arrays can be used for static as well as dynamic 3D cell cultures. The microcavities can be manufactured with pores ranging from a few nanometers to several micrometres so that popular assays, such as transwell assays or assays with active medium flow, can be realised. One of the standard formats, the 96-well microtiter plate, is able to generate 16.224 (96 x 169) 3D aggregates in a single experiment. Because the design of the array is self-referencing and repetitive, the position of each of the microcavities, and thereby the 3D-aggregate, is known and fixed making it very easy to find and retrieve single aggregates during long-term experiments (**Figure 13**). The production process allows many materials to be processed such as tissue culture polystyrene, polycarbonate, cyclo-olefin-polymer, cyclo-olefin-co-polymer and fluorinated ethylene propylene, etc. Moreover, the polymer surface can be modified so that the platform can be used for easy and rapid spheroid generation without the need of an aggregate transfer. Microcavity arrays have successfully been used for kinase profiling, embryonic and adult stem cell differentiation, toxicity assays and more. Therefore, it is believed the MTP-based microcavity array platform is an ideal high-throughput/high-content screening assay tool.

Agilent Technologies (www.agilent.com) has developed a 96-well spheroid microplate which enables measurement of cellular energy metabolism (oxidative phosphorylation and glycolysis) in single spheroids using the Seahorse XF analyser. This system provides a metabolic signature based on a set of fluorescent sensors that measure oxygen consumption and proton production within a small transient micro-chamber. Metabolism is a critical component of disease pathology and is highly sensitive to micro-environmental factors, phenotype and cell morphology. These signatures provide valuable insight into the pathology of disease and can be used to elucidate a better understanding of drug interaction, toxicology, immune response, and phenotype changes due to differentiation and muta-

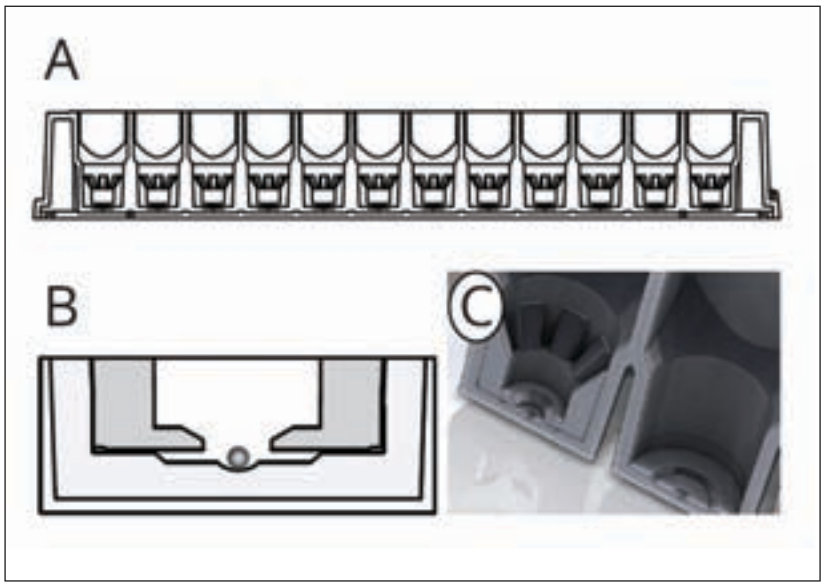


Figure 14: Schematic of an Agilent Technologies Seahorse XFe96 Spheroid Microplate showing: 1) unique geometry; 2) the correct position of the spheroid at the bottom of the well. The plate is designed to hold spheroids between 200 and 500µM in diameter; and 3) the venting system that allows the optimal media mixing around each spheroid

tion. To date, several studies have shown significant differences in response to drug interaction between 2D and 3D models. For example, A549 cells prepared as both 2D cultures and 3D spheroids show significant differences in response to an anti-cancer chemotherapy drug (Pemetrexed). These experiments were carried out in a single assay plate with multiple time points of exposure and drug concentrati on. In other experiments hepatocellular carcinoma cell line HepG2 spheroids were used to access mitochondrial impairment in response to drug-induced liver injury (DILI). The workflow for the Seahorse assay is highly flexible and conducive to running experiments with multiple tissue types, co-cultures, organoids and tissues (**Figure 14**).

The Cultrex® range from **AMSBIO** (www.amsbio.com) is based around a set of extracellular matrices optimised for different applications, allowing the researcher to direct cell activities by controlling their microenvironments. Cultrex® spheroid invasion and proliferation/viability assays use a dilute (non-gelled) ECM solution to stimulate the formation of cohesive spheroids in low adhesion environments. Such spheroids can be embedded into a gelled 'invasion matrix' to model cell invasion from the tumour spheroid. Human mesenchymal stem cells (hMSCs) and vascular cells can be added to model the role of stroma and vascular tissue respectively in a 3D 'triculture' model, producing microtumours that exhibit tumour morphology and drug response similar to published xenograft data, thus demonstrating a more physiologically predictive *in vitro* model (**Figure 15**). The Reduced Growth Factor format of Cultrex® Basement Membrane Extract 2 (BME 2) extracellular matrix has been shown to work well for growing organoids; especially using techniques based on the LGR5 stem cell marker/Wnt signalling system, pioneered by Hans Clevers and co-workers. More recently, an additional formulation of Cultrex® BME has been developed, known as BME R1. This matrix provides a proprietary formulation that has higher tensile strength when compared to other Cultrex® matrices: BME 1, BME 2 and BME 3. BME R1 has a higher concentration of entactin, one of the BME components that connects laminins and collagens, reinforcing the hydrogel structure. BME R1 is recommended for use with 'difficult to grow' organoid cultures.

Researchers have been using **Corning** (www.corning.com/lifesciences) Matrigel® matrix and Transwell® permeable supports for more than 25 years to mimic the 3D surface that cells naturally grow on. Building on this pioneering experience, Corning has introduced new surfaces and tools for manual and high throughput 3D cell culture, including 96-well and 384-well spheroid microplates and HTS Transwell® tissue culture systems. Corning spheroid microplates have round

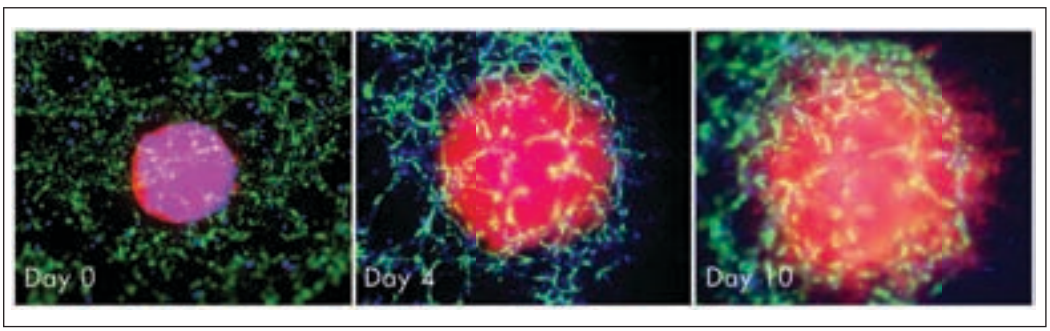


Figure 15
Tricultures of Multicellular Tumour Spheroids, human Mesenchymal Stem Cells (hMSC) and endothelial tubules in Cultrex® Basement Membrane Extract 2 (BME 2) from AMSBIO produce physiological breast cancer niche with tumour growth, tumour invasion and endothelial recruitment. Image shows formation of tri-culture structure over 10 days. Red: breast cancer cell line (MDA-MB-231), Green: human umbilical vein endothelial cells (HUVECs); Blue: human adipose-derived mesenchymal stem cells (hMSCs)

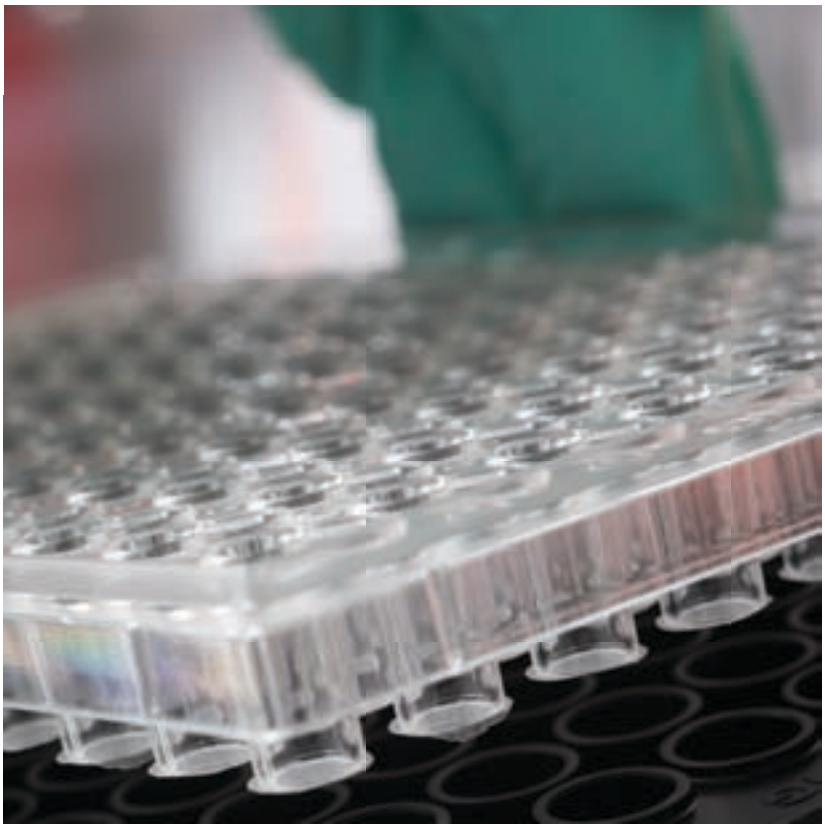


Figure 16: Corning's 96-well spheroid microplate and HTS Transwell®-96 tissue culture system

well-bottom geometry and are coated with Ultra-Low Attachment surface, resulting in the formation of highly reproducible, single multi-cellular tumour spheroids, centred in each well. Corning has recently demonstrated a 3D model to study cancer/immune cell interactions by combining its 96-well spheroid microplates and HTS Transwell-96 tissue culture system. Corning HTS Transwells-96 permeable support systems are commonly used for drug transport and migration/invasion studies. By replacing the standard 2D flat-bottom Transwell receiver plate with a Corning spheroid microplate, the ability to investigate items such as immune cell homing, tumour cytotoxicity and tumour immune evasion in an easy-to-use, 3D, high throughput assay is possible. Later this year, Corning plans to introduce a 1536-well spheroid microplate for ultra-high throughput screening. As more labs transition from 2D to 3D cell culture, the company also provides expert technical support for its customers who are either just starting out, or exploring more advanced techniques such as 3D HTS (**Figure 16**).

Figure 17: 96-well round bottom Greiner Bio-One CELLSTAR® microplate with cell-repellent surface to enhance the formation of a single spheroid per well

Greiner Bio-One (www.gbo.com/bioscience) has expanded its CELLSTAR® line with a cell-repellent surface to address the specific needs in 3D cell culturing. Vessels with a cell-repellent surface prevent effectively cell adherence and offer a straight-forward, easy-to-handle solution for 3D cell culture. The cell-repellent product portfolio comprises microplates ranging from six up to 384 wells, various dishes and cell culture flasks. Round-bottom microplates drive the formation of a single spheroid per well, whereas cultivation in flat-bottom plates results in the formation of multiple spheroids. In addition, microplates with cell-repellent surfaces are an ideal platform for gel-based 3D cultures, as they prevent the formation of adherent 2D subcultures. The cell-repellent effect is achieved through an innovative chemical surface modification. In applications such as microscopic analysis of single spheroids with high magnification, where perfect optical properties are requested, Greiner Bio-One collaborates with Nano3D Biosciences (n3D) to offer an additional technology for 3D cell culture: magnetic levitation and magnetic bioprinting. The n3D core technology is based on magnetic nanoparticles coated with PDL to magnetise cells by attaching to the cell membrane. The application of weak magnetic forces induces the aggregation of the magnetised cells, either by levitation or printing, to form structurally and biologically-representative 3D models *in vitro*. Magnetised spheroids are kept in place, while liquids are added or removed with almost no loss of spheroids. Various studies have demonstrated that the magnetic particles do not show any effect on the growth characteristics and physiology of cells (**Figure 17**).

InSphero (www.insphero.com) specialises in 3D cell culture technologies for toxicology, diabetes and cancer biology. The only global supplier of 'assay-ready' microtissues and 3D-cell-culture-based screening services, InSphero leverages its patented hanging drop and ultra-low attachment plates and proprietary 3D Select™ Processes for development and industry-scale production of standardised scaffold-free 3D InSight™ Microtissues. The company offers a broad range of fit-for-purpose liver, pancreatic islet and tumour models certified for *in vitro* safety testing, disease modelling and drug discovery applications. InSphero human liver microtissues are co-cultures of pooled primary human hepatocytes and Kupffer cells, and provide a scalable, highly-sensitive and specific model for drug-induced liver injury (DILI) testing. Likewise, human islet microtissues provide a more reproducible and convenient *in vitro* diabetes model by eliminating size and cellular heterogeneity and laborious hand-picking associated with using native human islets. InSphero's process normalises the endocrine cell composition, providing long-term, robust functionality which is pre-qualified and rigorously QCd with each production lot. Microtissues are delivered in a user-friendly GravityTRAP™ plate designed for safe microtissue handling or contract screening services using 3D InSight™ microtissues are also available. InSphero continues to drive innovation in the field of 3D cell technologies, and recently launched the GravityTRAP™ 384 Microtissue Platform optimised for 3D applications using high content imaging systems. This new high-throughput screening platform features a unique well design to prevent accidental aspiration of microtissues during medium exchange. 3D InSight™ Microtissues will be available in the 384-well platform this year, beginning with liver models for early-stage DILI screening and custom tumour models for oncology research (**Figure 18**).

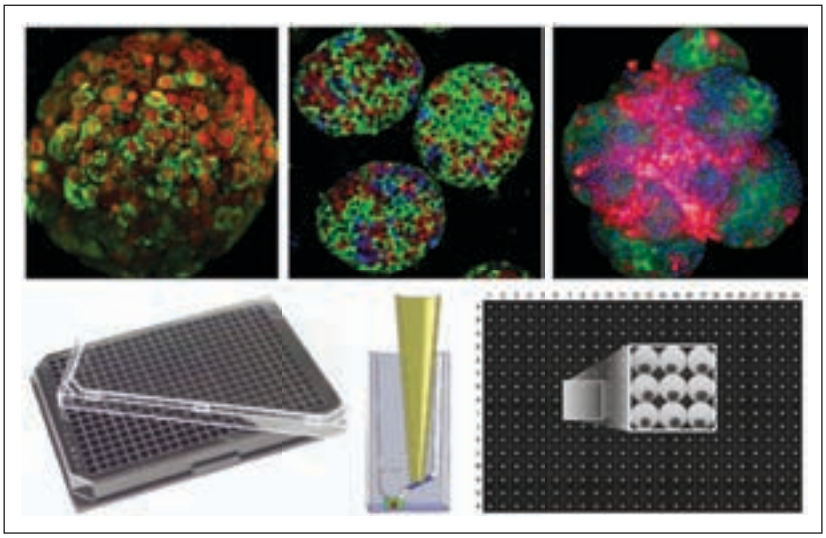


Figure 18: InSphero supplies 3D microtissues and screening services for drug discovery and development in high-throughput, automation compatible formats. Pictured: Liver (top left), islet (top centre) and tumour (top right) microtissues, and the GravityTRAP™ 384 Microtissue Platform (bottom) for 3D screening and imaging applications

The Elplasia™ 3D Spheroid Generator (www.elplasia.com) is a product line of **Kuraray Co Ltd**. The Elplasia™ Spheroid Generator's newest product is the Elplasia™ Multiple-Pore-Circular (MPc), a device with micro-holes that is designed to be nested within plates. MPc is six devices that fit within one plate. Using this insert design, cell suspension is added across the top of the insert where cells are free to fall into the 650 micro-holes in the insert device. Spheroids form without further manual manipulation, which can then remain in the plate to undergo differentiation. Upon completion of the desired process, spheroids can easily be harvested from the insert by filling the wells below with appropriate media and touching off the insert to the media. MPc eliminates complications by offering a method to create a large numbers of spheroids from the micro-hole s of the insert device. The device format is compatible with manual or automated liquid

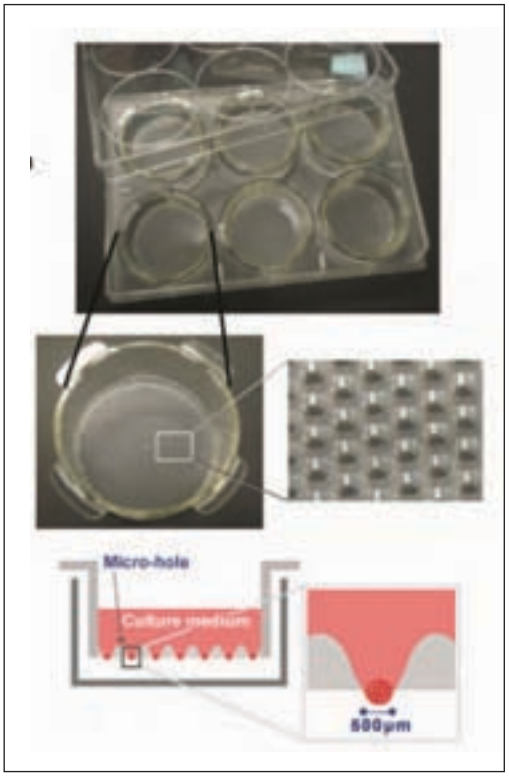


Figure 19
The Elplasia™ 3D Spheroid Generator from Kuraray. Top panel: 6-well MPc plate containing 500μm micro-hole inserts; Middle panel: close-up view of the insert device and the micro-holes; Bottom panel: sectional view of the insert in the plate containing medium showing micro hanging drops, containing spheroids, forming in each micro-hole

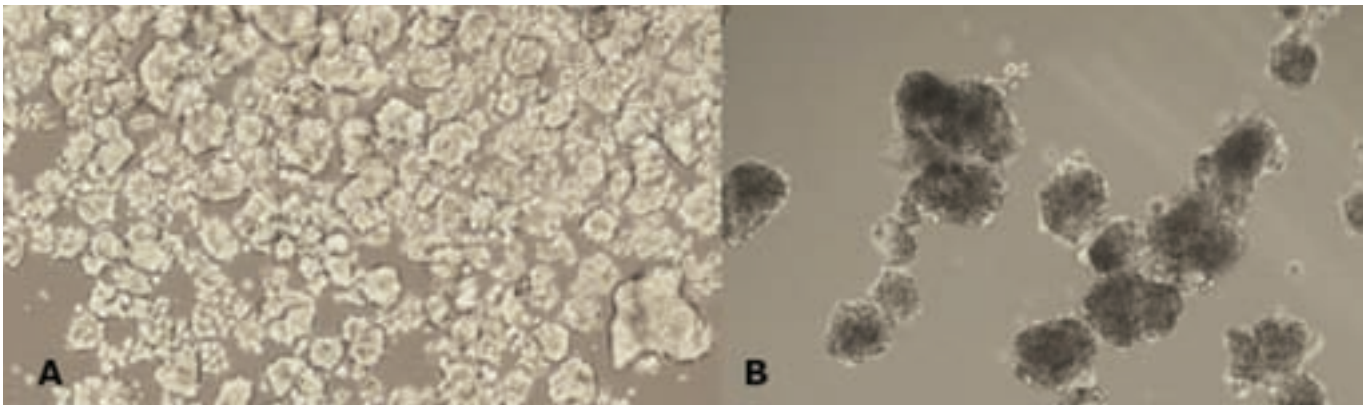


Figure 20: MilliporeSigma 3dGRO™ Spheroid Medium allows for the efficient generation and serial passaging of three-dimensional tumourspheres in various cell lines including MCF-7 breast (A) and E006AA prostate (B) cancer cell lines. This new serum-free defined media allows for the enrichment of cancer stem cell populations in 3D spheroid cultures

handling equipment. Also, you can order different micro-hole sizes. Products complementary to the Elplasia™ Spheroid Generators are available for mass spheroid culture, micro tissue engineering and tissue reconstruction along with other applications outlined on its website (**Figure 19**).

MilliporeSigma (www.milliporesigma.com) is dedicated to providing researchers with the latest innovations in 3D models and tools. It offers a panel of spheroid-adapted technologies from high content dedicated systems to low throughput research-based applications. Corning® Spheroid Microplates are particularly appropriate for HCS/HCS spheroids studies, as illustrated in a recent publication where these plates were used to measure the effects of Doxorubicin on 12 different cancer cell lines, screened on their ability to maintain a regular spheroid shape, in real-time[2]. Depending on application and downstream analysis, however, approaches used to generate and study spheroids will be different. For example, it has been shown that the hanging drop system is a well-suited technology for imaging a spheroid's diameter and shape, known to be critical parameters to consider for generating accurate results. Perfecta3D® Hanging Drop Plates have been recently used for this purpose to establish and characterise a method for 3D spheroidal growth of liver cancer cell lines for five different cell lines[3].

Figure 21
ORGANOGENIX scaffold-embedded U pattern TAKOYAKI plate for promoting self-organisation and providing uniform and size-controlled spheroids

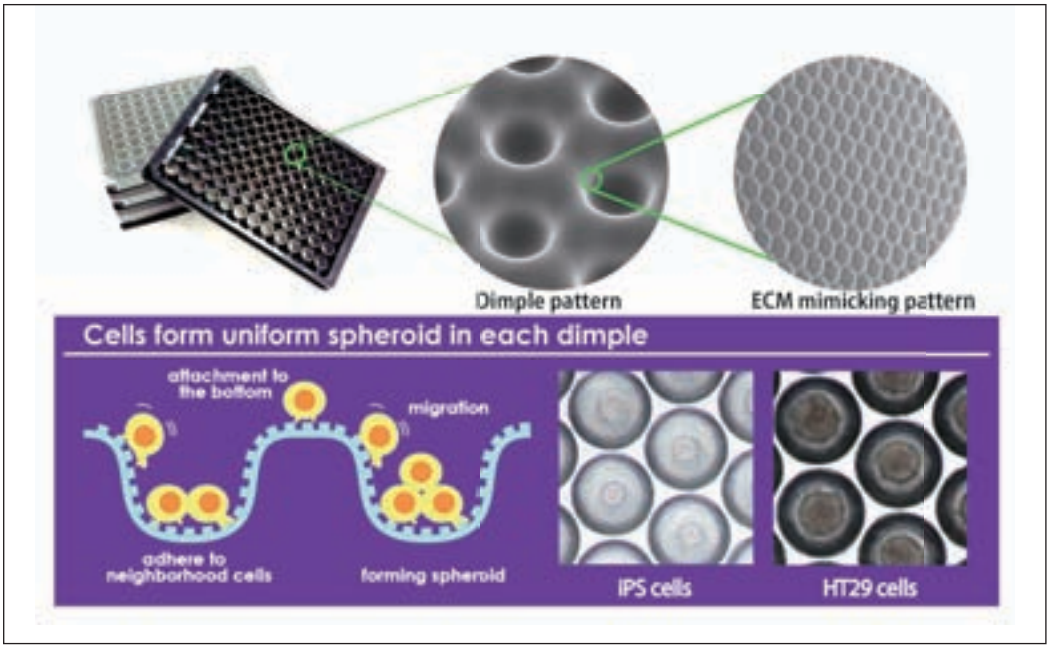


Figure 22: PerkinElmer's spheroid plate offering (left-to-right): CellCarrier® ULA plate, GravityTRAP™ ULA plate and GravityPLUS™ Hanging Drop Plate

Technologies also need to adapt to new research challenges. Tumoursphere (spheroids composed of tumour stem cells) culture is an emerging model for studying and expanding the cancer stem cell (CSC) population. CSCs and their niche cells are important for tumour progression and metastasis, but specialised reagents are requested to allow CSC proliferation. MilliporeSigma is now offering a new chemically-defined, serum-free medium (3dGRO™ Spheroid Medium), that ensures stable tumoursphere formation and proliferation, but also increased ALDHHigh stem cell population (**Figure 20**).

ORGANOGENIX (www.nanocultureplate.com), previously SCIVAX Life Science, develops and sells 3D cell culture and organoid culture products with MBL International. The NanoCulture Plate (NCP), one of ORGANOGENIX's main products, is engineered with an evenly patterned nanoscale structure on a special plastic film attached to the bottom of the plate/dish. This pattern mimics the extracellular matrix and acts as a scaffold for cells, supports cells to migrate across the plate and form healthy spheroids. The pattern eliminates the need of biological/gel matrix, controls the adhesion of cell to the film and enhances cell proliferation while cells form spheroids. This gel-free scaffold type 3D platform has some advantages, ie high experimental reproducibility, usability for high-throughput screening and high-content imaging. There are more than 80 publications referring to NCP so far. Most of them are related to cancer research, with a wide assortment of applications and studies, such as signalling, hypoxia, anti-cancer drug sensitivity screen, proteomic analysis, live imaging by using time-lapse imaging apparatus, etc. NCP is also used for toxicology or regenerative medicine research. ORGANOGENIX has also developed a new-generation NCP, called the TAKOYAKI plate, which has multiple semi-micro dimples with a nano-grid structure on the bottom (**Figure 21**). This new plate will provide a stricter 3D cell culture, eg control of spheroid size, array of spheroids in the well and frequent medium change. The nano-grid acts as a scaffold and promotes self-organisation. This plate is suitable for organoid culture, drug sensitivity assays, drug permeability assays and iPS differentiation assays.

Culturing cells in 3D enables more natural cellular interactions and can mimic the physiologically relevant *in vivo* microarchitecture. The use of 3D cell culture systems can therefore reduce downstream costs such as secondary assays as well as *in vivo* animal testing by providing more predictive data earlier. **PerkinElmer** (www.perkinelmer.com) offers a broad range of solutions for the culture, handling, detection and analysis of multicellular spheroids. The choice of specialised microplates for 3D cell culture will depend on the cell types used and the readout needs of the assay. CellCarrier® Spheroid ULA 96-well microplates feature a unique Ultra-Low Attachment (ULA)-coating that enables consistent formation of round spheroids from numerous cellular models. This coating suppresses satellite spheroid formation to enable easier analysis and more reproducible results. PerkinElmer also provides InSphero's 3D GravityTRAP™ ULA plate for long-term culture and the GravityPLUS™ Hanging Drop 96-well system for advanced assays that require more size consistency or defined co-cultures with primary cells. Such experiments can be automated using a PerkinElmer® Zephyr® G3 workstation to simplify liquid handling and reduce seeding variability originating from manual pipetting. Spheroid growth can be rapidly quantified by imaging with the EnSight® Multimode Plate Reader, which also reads viability endpoint assays such as ATPlite™ 3D or measures secreted proteins from supernatant using AlphaLISA® technology. A more detailed understanding of spheroid organisation and function, eg bile caniculi formation in liver spheroids can be achieved after confocal imaging with the Opera® Phenix™ or Operetta® CLS™ High-Content Analysis Systems and analysis with Harmony® software (**Figure 22**).

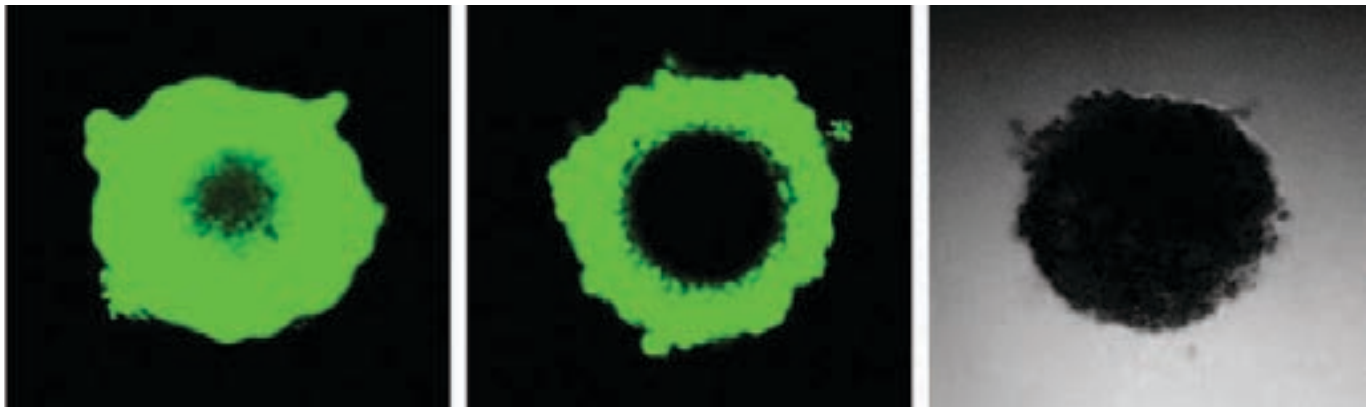


Figure 23: HEK293 cell spheroids were grown using the GravityPLUS™ hanging-drop plate (InSphero AG). CellTox™ Green Dye was combined with the Promega CellTiter-Glo® 3D Reagent and added to the spheroid in the left image. A competitor's ATP detection reagent combined with CellTox™ Green was added to the spheroid in the centre image. The samples were shaken for 5min and incubated for 30 minutes total before imaging using confocal laser microscopy. Green staining indicates cell membranes have been disrupted and shows the CellTiter-Glo® 3D Reagent is more effective at lysing cells (left panel). The right panel shows a differential image contrast image of a spheroid treated with CellTiter-Glo® 3D Reagent indicating even though cells have been lysed, the overall structure remains intact

There is an unmet need to validate 3D cultures and the performance of assays on 3D spheroids. Most assays available have been designed for use with cells grown as a monolayer in 2D. A major difference between 2D and 3D culture models is the distance an assay reagent must penetrate to access all of the cells. A 2D monolayer of cells grown on plastic is typically less than 10µm thick, whereas a 200µm diameter spheroid presents at least a 10-fold increase in the distance a reagent must penetrate to reach all the cells. **Promega** (www.promega.com) has used three approaches to adapt assays for use with 3D spheroids: reformulation to improve the ability to lyse cells; modifying the assay protocol; or using a small molecule probe reagent that is able to penetrate 3D structures. To address this problem, we have reformulated the original version of our ATP viability assay to create the CellTiter-Glo® 3D Cell Viability Assay designed for use with 3D culture models. The new reagent formulation has an improved capacity to lyse cells in large spheroids and a protocol that includes increased physical disruption of 3D spheroids by incorporating more rigorous sample shaking and increased incubation time in the presence of the lysis solution (**Figure 23**). The performance of CellTiter-Glo® 3D Cell Viability Assay has been validated with a range of cell types and spheroid sizes by comparison to acid extraction, which is the gold standard method for extracting ATP from tissue samples. For apoptosis detection, to maintain and extract caspase-3/7 activity, and achieve lysis of cells in large spheroids, Promega has designed a modified assay protocol that incorporates longer sample shaking and incubation times with its Caspase-Glo® 3/7 Assay in the presence of the lytic reagent. Examples where small molecule probes penetrate deep into 3D structures and serve as an alternative to overcome the hurdle of lysing large spheroids include the use of DNA binding dyes (eg CellTox™ Green Cytotoxicity Assay) to detect dead cells and probes to detect viable cells such as the approach used with the RealTime-Glo™ MT Cell Viability Assay.

Figure 24: STEMCELL's range of products for 3D spheroid culture includes AggreWell™ plates for generating multicellular 3D spheroids and specialised media and protocols for 3D organoid models, including IntestiCult™

To support the fast-growing field of 3D culture, **STEMCELL** (www.stemcell.com) offers a portfolio of products for 3D spheroid models of varying complexities. On the simpler end of the 3D spectrum, AggreWell™ plates can be used to generate multicellular spheroids. The microwells-in-wells design provides an easy way to generate large numbers of highly uniform multicellular spheroids,

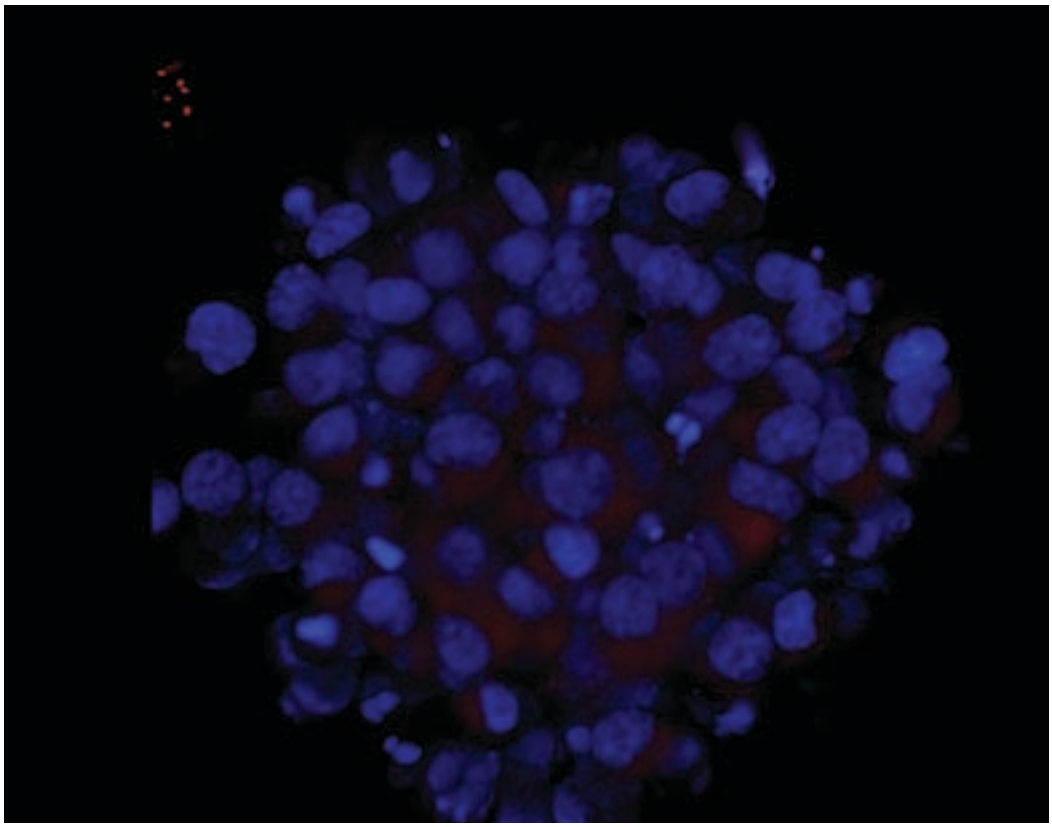


Figure 25
A single HeLa spheroid used in the assessment of the hypoxic cores in HeLa spheroids. After two days of culture on Thermo Scientific Nunclon Sphera 96-well U-bottom plates, the HeLa spheroids were stained with Image-iT Hypoxia Probe (red) and NucBlue Live ReadyProbes Reagent (blue). The images were taken on a confocal microscope

including embryoid bodies (EBs) for pluripotent stem cell differentiation, and cancer spheroids for tumour modelling or drug discovery research. To culture spheroids generated in AggreWell™ or formed from stem/progenitor cells, STEMCELL provides specialised cell-specific media, including MammoCult™ (mammary and breast cancer cells), NeuroCult™ (neural stem and progenitor cells, including brain tumour stem cells) and PneumaCult™ (upper airway, including bronchial, nasal and tracheal cells). To support applications requiring more complex 3D models, STEMCELL provides specialised culture media and optimised protocols for organoids. Organoids are small 3D clusters of cells that represent 'mini-organs' in structure and function. IntestiCult™ medium can be used to derive and culture organoids from small and large intestinal epithelium. These intestinal organoids or 'mini-guts,' are physiologically relevant and feature a polarised epithelium that contains all the known cell types of the adult intestinal epithelium. IntestiCult™ medium was commercialised under exclusive licence from The HUB Foundation for Organoid Technology. Specialised media to support organoid culture from other tissues are in development, including cerebral organoids or 'mini-brains' and gastrointestinal organoids (exclusive licensing agreements with Institute of Molecular Biotechnology (IMBA) and Cincinnati Children's Hospital, respectively) (**Figure 24**).

Driven by the mounting evidence that 3D cell cultures are more physiologically relevant than traditional monolayer cultures, 3D culture systems are emerging as the preferred *in vitro* platform for disease modelling and drug discovery applications. While multiple scaffold-based 3D culture systems have been around for decades, more researchers have set their eyes on the high throughput-friendly scaffold-free 3D culture systems. The **Thermo Scientific** (www.thermofisher.com/order/catalog/product/174925) Nunclon™ Sphera cell culture plate features an extremely easy-to-adapt scaffold-free culture surface that promotes the self-assembly of 3D cell aggregates. The vital combination of the cell-repellent coating and the proper curvature of the well effectively produces a single spheroid in each well with consistent shapes and sizes, which conveniently enables researchers to evaluate cell health and cellular responses *in situ* by fluorescence- and colorimetric-based assays. The development of such 3D culture systems offers particular benefits in cancer biology where the spheroids

share several key histomorphological and functional traits with those of the solid tumours[4,5]. For example, the evidence of a hypoxic core in the cancer spheroid cultured on Nunclon Sphera plate is demonstrated (**Figure 25**) by leveraging the Image-iT™ Hypoxia Probe from Thermo Fisher Scientific, a highly oxygen-sensitive compound that measures hypoxia in live cells using real-time oxygen detector with reversible fluoregenic response. The gradient of oxygen in spheroids, progressing from normoxic cells at the periphery to hypoxic cells at the core, provides an excellent model for assessing novel pharmacological agents and drug delivery methods.

Discussion

There seems to be little doubt that cells in 3D form offer more natural cellular interactions and mimic physiologically relevant *in vivo*-like microarchitecture. There is also increasing evidence that they may elicit a differential response to drugs or stimuli than traditional 2D monolayer cultures, ie in 3D they may have different metabolic signatures. Scaffold-free 3D culture systems that generate spheroids (and other similar multicellular aggregations) have in recent years solicited significant interest from researchers seeking ease of entry into 3D culture and a route to transition into plate-based higher throughput. The emergence of ULA or cell-repellent plates (mainly from Corning and Greiner Bio-One) has been a major contributor in promoting this trend and establishing a momentum behind spheroids that has fostered many additional developments and innovation. ULA and cell repellent plates are currently the most popular approach to the generation of a single spheroid per well, but other spheroid plate formats may be better suited in creating multiple spheroids, for enhanced size uniformity, for specific cells types or for niche applications. It is probably fair to conclude that spheroid generation is no longer an obstacle and the emphasis has shifted more to improving size and shape consistency, defined co-cultures with primary cells and optimising conditions for those 'difficult to grow' cells. Some of the new developments reported by vendors in this article included: microcavity arrays (300Microns); combining ULA plates with other existing microplate compatible products (Transwells) to support novel applications in 3D (Corning); redesigning the well shape to prevent accidental aspiration of microtissues during medium exchange (InSphero, PerkinElmer); a plate insert with micro-hole hanging drops that facilitates generation and harvesting of large numbers of uniform spheroids (Elplasia); the development of spheroid optimised assay reagents (Promega, Thermo Fisher Scientific); a system for measurement of cellular energy metabolism in single spheroids (Agilent); the use of ECM solution to stimulate the formation of spheroids (Amsbio); and new specialised culture media for organoids and tumourspheres (MilliporeSigma, STEMCELL). Other vendors report the evolution of their spheroid-generating formats to new and improved second-generation products (Organogenix and STEMCELL), a sure sign that spheroids are gaining traction. Feedback from the survey reported that just under 50% of respondents have a specific requirement to transfer spheroids out of their original culture vessel (eg microplate well) and that there is still an unmet need for devices or mechanisms to perform this transfer of spheroids without any cell damage or disaggregation that may arise from the manipulation.

In conclusion, much progress has been made in recent years in the supporting literature, culture tools, detection technologies, reagents and service offerings enabling spheroids to be adopted as the preferred *in vitro* 3D platform in safety testing, disease modelling and drug discovery applications. It should not be overlooked that spheroids are also proving invaluable in regenerative medicine/tissue engineering research where they are used as a biological unit or building block to merge into a more complex tissue or organ structures. DDW

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