3D CELL CULTURE

easier said than done!

The transition from cell culture on the flat surface of a conventional two-dimensional (2D) culture vessel to a three-dimensional (3D) environment, matrix or scaffold with 3D architecture has begun, and is providing much needed support for emerging applications in tissue engineering and stem cell research. Biomimetic scaffolds (eg hydrogel or collagen) have shown potential in culturing specific cell types and in investigating different aspects of the cell-matrix interaction in 3D. Structural scaffolds, made from the same material as 2D plate surfaces (ie polystyrene), would seem to be compatible with many routine (easy) 2D assays. Microfluidic devices with moulded microchannels incorporating biomimetic scaffolds are now available to support specific 3D applications, eg invasion assays and specific tumour cell models. Systems directly supporting the automation of 3D cell culture and/or tissue creation are beginning to emerge and should facilitate the scale up of cell production, but also impact how 3D generated cells are used in drug screening assays and how organs and tissues can be consistently produced. The prospect of developing more physiologically relevant 3D models systems for use in *in vitro* toxicology is particularly compelling. However, the state-of-the-art is still some way off from providing fully validated or robust 3D culture solutions and tools and the field remains open to major improvements at this point in time.

By Dr John Comley

Increasingly the term three-dimensional (3D) is being applied in relation to cell culture. Simplistically this involves growing cells in a 3D environment, matrix or on a scaffold with 3D architecture as opposed to the flat surface of a conventional two-dimensional (2D) culture vessel. However, what 3D cell culture encompasses is hard to define and varies widely depending upon the application. In most drug discovery areas and in stem cell research we are mainly talking about anchorage-dependent cell culture on a 3D scaffold, which can in the least demanding cases be made of the same material as the 2D surface, although often more complex biological gels or coatings are required. In tissue engineering and clinical research, and in some aspects of safety assessment and the delivery of stem cells, the focus is on actual organ and tissue development. In an attempt to bring some clarity to the subject, HTStec undertook a survey and report on 3D cell culture in February 2010[1].

Main advantages of 3D cell culture

To better understand what is driving the investigation of 3D cell culture, the survey first sought to identify what were perceived as the most important

Figure 1: Most important advantages of 3D cell culture

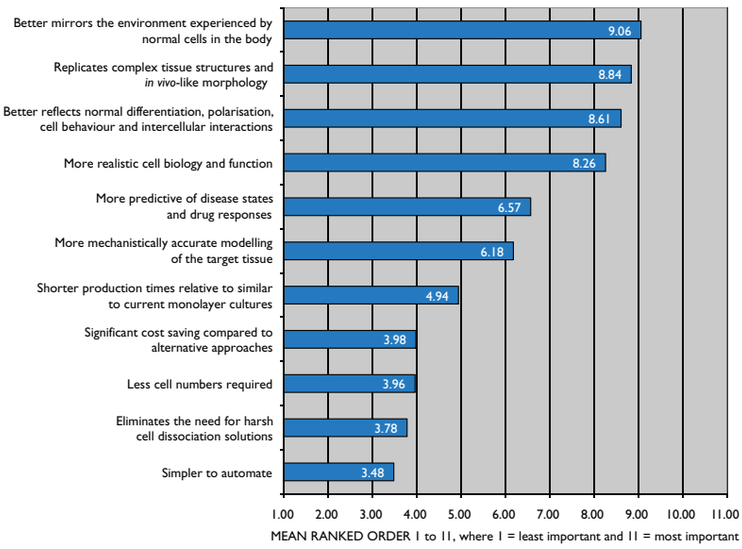
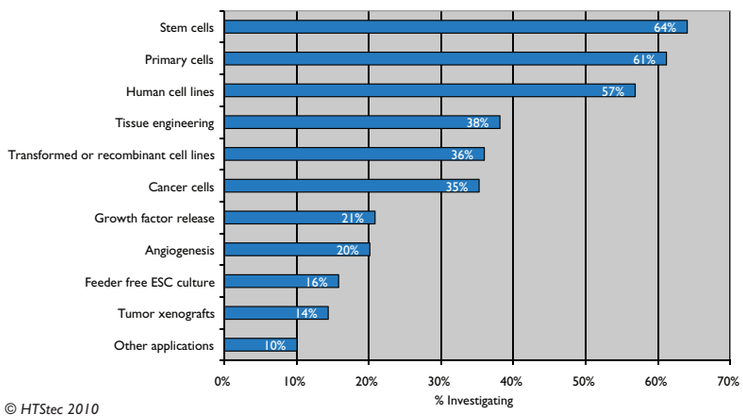


Figure 2: Main applications for 3D scaffolds/formats investigated



advantages of 3D cell culture. This revealed that better mirrors the environment experienced by normal cells in the body was the most important advantage to survey respondents. In the ranking this was closely followed by replicates complex tissue structures and *in vivo*-like morphology; and then better reflects normal differentiation, polarisation, cell behaviour and intercellular interactions. Ranked least important advantages were simpler to automate and eliminate the need for harsh cell dissociation solutions (**Figure 1**).

Main application for 3D scaffolds/formats

The main application for the 3D scaffolds/formats (ie the substrates on which cells are cultured) under investigation by survey respondents are presented in **Figure 2**. By application here we include both the cell origin and the desired outcomes. This analysis showed that the majority (64%) was investigating stem cells, this was closely followed by primary cells (61% investigating) and human cell lines (57% investigating). Other important applications were investigated by fewer respondents, eg tissue engineering (38% investigating) and cancer cells (35% investigating).

Types of primary cells most investigated for 3D cell culture

The type of primary cells most investigated by survey respondents for 3D cell culture was fibroblasts, this followed by endothelial cells, mesenchymal stem cells, and then hepatocytes (**Figure 3**).

3D scaffolds/formats that have shown most promise

The 3D scaffolds/formats that have shown most promise in 3D cell culture was gel/hydrogel, this was followed by ECM (extra-cellular matrix) sheet, aggregates/spheroids and then collagen tissue constructs (**Figure 4**).

Where 3D cell culture will impact the most

Survey respondents ranked tissue/organ engineering as the area where they expect 3D cell culture to impact the most over the coming years. This was followed by all aspects of basic research and then drug discovery application areas (**Figure 5**).

Assay types most successfully demonstrated with 3D cultures

The assay types survey respondents have most successfully demonstrated to date (2010) with 3D cell cultures were cell viability, closely followed by cell proliferation, then cell migration and cell signalling assays (**Figure 6**).

Transitioning from 2D to 3D cultures

Two-thirds of people surveyed plan to transition their cell culture from 2D to 3D, with half of these having already transitioned some part of their work to 3D. Greater biological relevance was ranked as the most important reason for transitioning from 2D to 3D cell culture. This was followed by enhanced cell viability/responsiveness and better quality of assay results (**Figure 7**).

3D automation

The ability to automate 3D cell culture should facilitate the production of large quantities of cells, but also impact how 3D generated cells are used in drug screening assays and how organs and tissues can be consistently produced. When survey respondents were asked about their use of automation in 3D cell culture today, most (86%) replied they were not yet using it and/or don't have a system, only 7% have already implemented changes to existing 2D automated cell culture systems to enable 3D, with a further 7% actively looking at or investigating 3D enhancements. In separate questions 43% of survey respondents indicated that the availability of automated equipment would influence their future choice of 3D scaffold/format; and 32% indicated that they expect to be able to use existing equipment to scale up and automate their chosen 3D scaffold/format.

Latest developments in 3D cell Culture

The following vendor snapshots provide additional details and describe some of the latest developments in 3D cell culture, tissue generation and related automation:

Inspired by the principle that tissue engineering scaffolds can be used to create 3D *in vitro* tissue and organs, **3D Biotek** (www.3dbiotek.com) has developed two 3D cell culture product lines, the biodegradable scaffold, 3D Insert™–PCL, and non-degradable scaffold, 3D Insert™-PS. With the proprietary 3D Precision Microfabrication Technology, 3D Biotek can produce 3D scaffolds with well controlled and 100% connected porous structure. The 3D Insert™-PCL scaffolds are made from biodegradable poly(γ-caprolactone) (PCL). These PCL scaffolds are 100% interconnected, have a very well controlled porosity, and are mainly designed for applications in tissue engineering and stem cell research. 3D Insert™-PCL has most

Figure 8: 3D Biotek 3D Cell Culture Kit which contains 24 96-well 3D Insert™-PS scaffolds

Figure 3:Primary cell types investigated using 3D cell culture

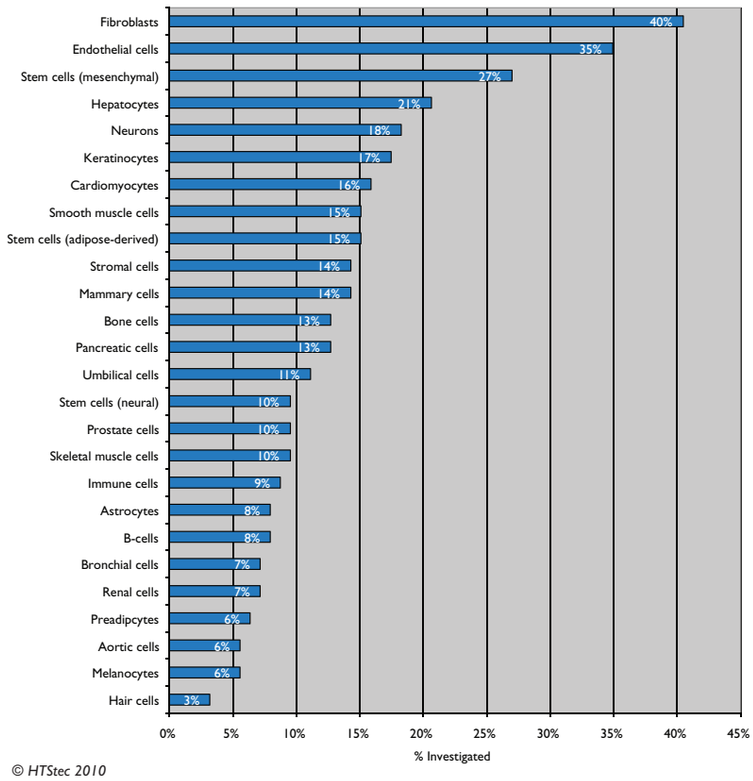


Figure 4: 3D scaffolds/formats that have shown most promise

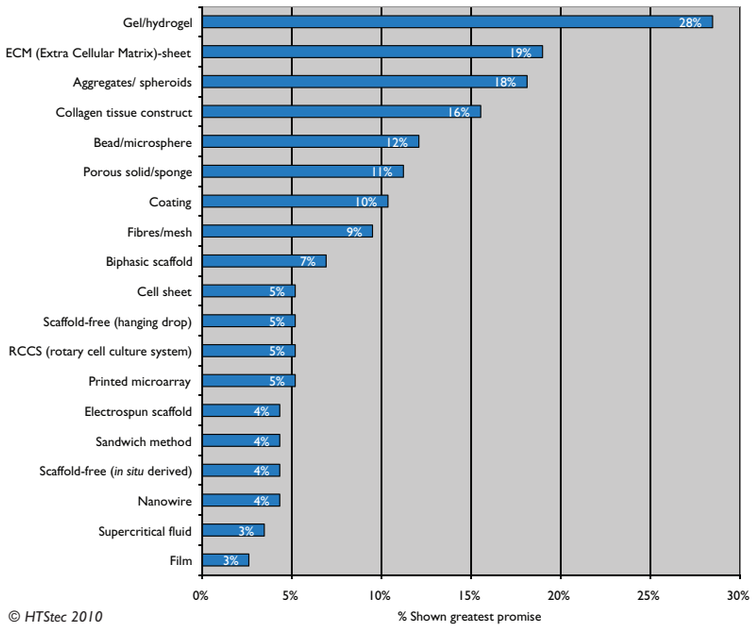


Figure 5: Where 3D cell culture is expected to impact most

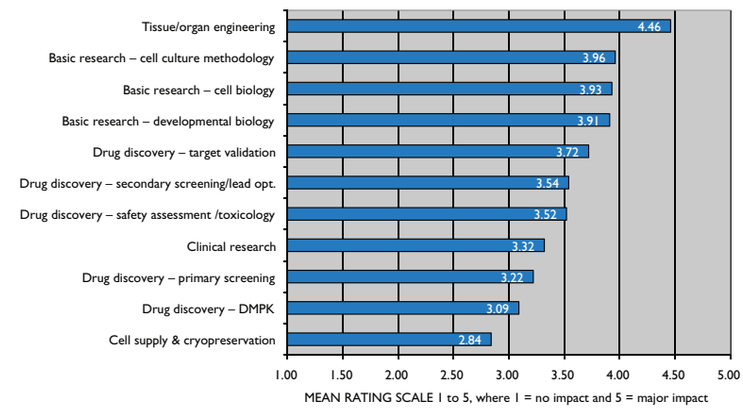


Figure 6: Assay types successfully used/ investigated in 3D cell culture

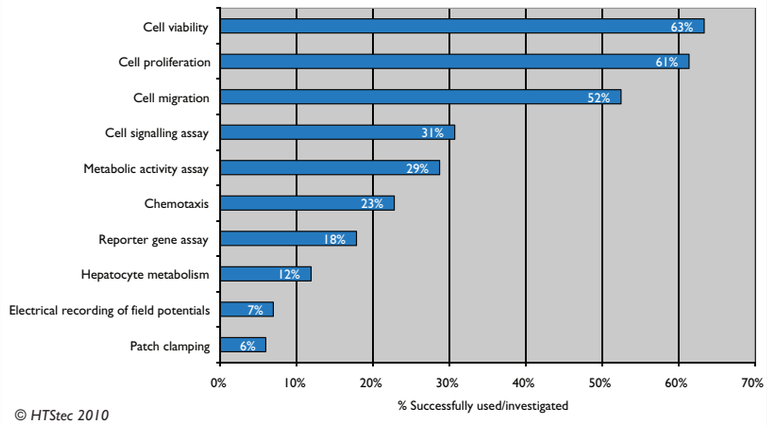
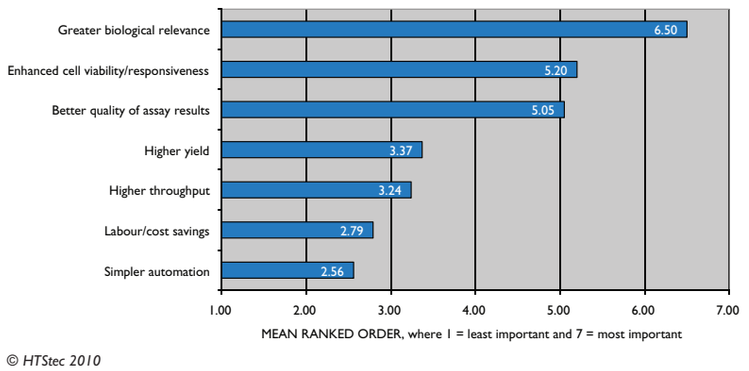


Figure 7: Most important objectives of transitioning from 2D to 3D culture



recently been chosen by The National Institute of Standard Technology (NIST) as the reference 3D tissue engineering scaffold. The non-degradable 3D Insert™-PS has been developed for conducting routine 3D cell culture. Made from the same material as traditional tissue culture plates, 3D Biotek has essentially engineered 2D polystyrene into a 3D scaffold. Combining the precisely engineered 3D structure with the inherent transparency of polystyrene material, creates an ideal 3D Insert™-PS scaffold for performing easy 3D cell culture. These PS scaffolds are compatible with most 2D assays and allow researchers to monitor 3D cell growth by simply using an inverted light microscope. 3D Biotek has initiated a partnership with BioCellChallenge SAS, a drug delivery company in France, and developed the world's first 3D cell transfection kit, which combines 3D Insert™-PS and Transfection Reagent into one kit. This is the world's first 3D *in vitro* transfection technology, which now allows researchers to achieve high delivery efficiencies of plasmid DNA into 3D cultured cells (**Figure 8**).

BellBrook Labs (www.bellbrooklabs.com) has developed a device and methods to address the need for automatable and high-content assays for

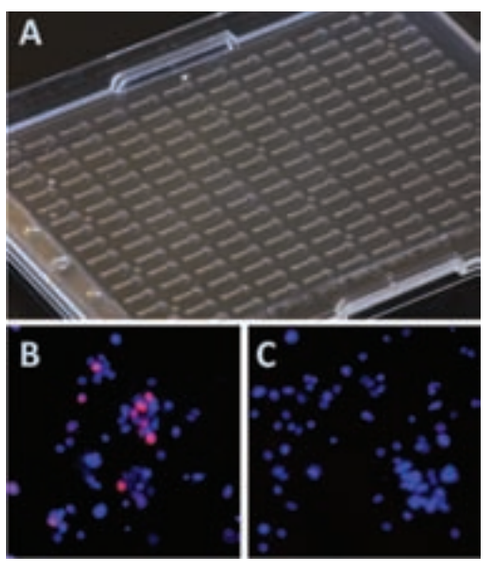


Figure 9: A) Schematic of BellBrook Labs IUVO MC5250 Microchannel. B&C) BxPC3 cells suspended in 3D Type I Collagen were incubated 18 hours and then stained for EdU incorporation using Invitrogen ClickIT EdU Imaging Kit. Hoescht staining of nuclei is shown in blue, and cells with incorporated EdU are shown in red. An average of 19% of untreated cells (B) have incorporated EdU, compared to 0% for cells treated (C) with 10μM Cycloheximide. n = 4, $p<0.001$

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pancreatic cancer cell models in 3D matrices. The device consists of an array of microfluidic channels specifically designed for the culture of cells in 3D format. For the pancreatic tumour cell model, BxPC3 cells were added to the channel suspended in neutralised Collagen Type I which traps the cells in 3D upon gelling. The channels are open at each end and allow for a droplet placed at one end to flow through the porous fibrillar collagen matrix to equilibrate the pressure across the channel. As opposed to well-based 3D culture, this simple addition and removal of droplets of growth media and assay reagents to the channels is done without disruption of the matrix and cells. After an overnight compound treatment, the cells were fixed, permeabilised and stained for EdU incorporation during DNA replication using Invitrogen's ClickIT Alexa 594 EdU Imaging Kit. Compounds that inhibit cell proliferation or are overtly cytotoxic have a lower incorporation of EdU. The channels are 140µm tall, which allows for imaging of the full depth of the 3D matrix with low power objectives. Here, (see **Figure 9**) we captured the channel region in one shot for each label (Hoescht and Alexa 594 EdU). Image analysis was performed with Metamorph's Multi-wavelength Cell Scoring application in which cells are first segmented by their nuclear Hoescht stain and then scored for EdU incorporation.

BioTek (www.biotek.com) is interested in the automation of cell-based assays, particularly in Global Cell Solutions GEM 3-D matrix as it simplifies the work flow of cell-based high throughput screening assays. Current cell culture techniques are two-dimensional (2-D) where cells attach to the microplate surface in a single monolayer. Trypsinisation is required to split cell cultures, and prepare them for downstream applications, which becomes increasingly difficult as scale-up is required for primary and secondary screens. 3-D cell culture using GEM, where cells grow on microcarriers suspended within the culture, eliminates the need for trypsinisation and enables the growth of high-density cultures in a small volume. Cells on the optically-transparent microcarrier can be dispensed directly from culture or frozen *in situ* to be thawed when needed without further culturing. **Figure 10** demonstrates the comparison of Histamine 1 antagonist pharmacology using GeneBLAzer® H1-NFAT-bla HEK 293T cells freshly cultured on GEM; frozen on GEM, then thawed; and using standard 2-D cell culture protocols. Receptor pharmacology was found to be equivalent across the three formats.

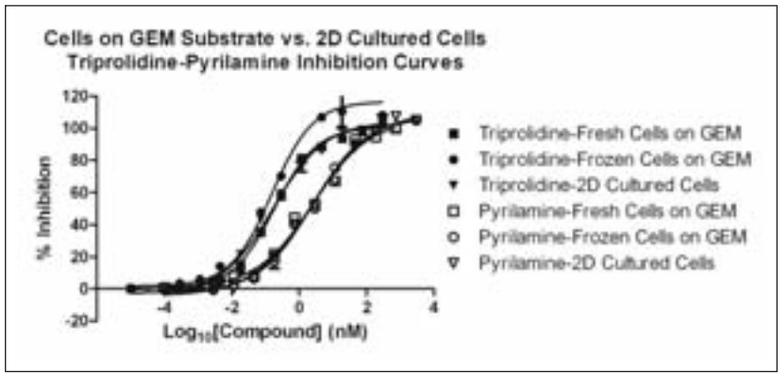

Figure 10: Comparison of Histamine 1 antagonist pharmacology using GeneBLAzer® H1-NFAT-bla HEK 293T cells. All assay steps were automated: cells and LiveBLAzer substrate were dispensed using the BioTek MicroFlo™ peristaltic pump dispenser; antagonist dose responses were serially diluted using the Precision™ automated pipetting station; and GeneBLAzer FRET signals measured using the BioTek Synergy™ 4 MultiMode Microplate Reader

CellASIC (www.cellasic.com/3D) has developed the 3D:M microfluidic plate, enabling long term perfusion culture of cells in a 3D environment. The microfluidics are integrated with a standard 96-well frame and do not require any external pumps, maximising compatibility with existing assays. Each perfusion unit consists of four well positions (inlet, culture chamber, cell/gel inlet, outlet) resulting in 24 independent units per plate. The user loads their selected cell/gel combination via capillary flow to fill the 150 nanolitre culture chamber. Different protocols allow for embedding cells in gel, overlay of gel on a layer of cells, or 2D culture without gel. The micro-chamber floor is a #1.5 thickness glass cover slide to facilitate high magnification microscopy. The cells are fed by gravity driven perfusion of medium, set to a rate of 40

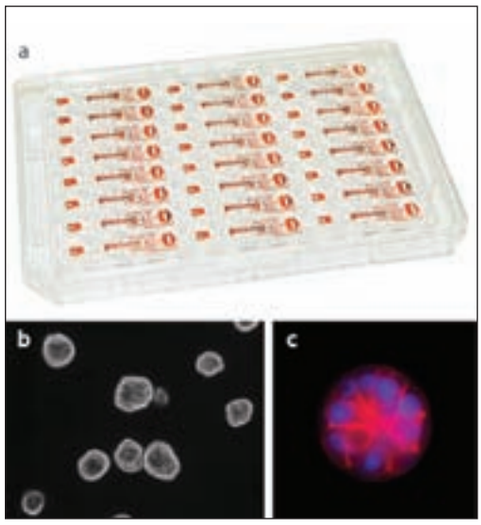

Figure 11
(a) The 24-unit CellASIC 3D:M microfluidic 3D perfusion plate. (b) MCF-10A breast epithelial cells cultured in Matrigel (BD Biosciences) for four days in the microchamber, and (c) actin staining highlights the acinar organisation of cells in 3D culture

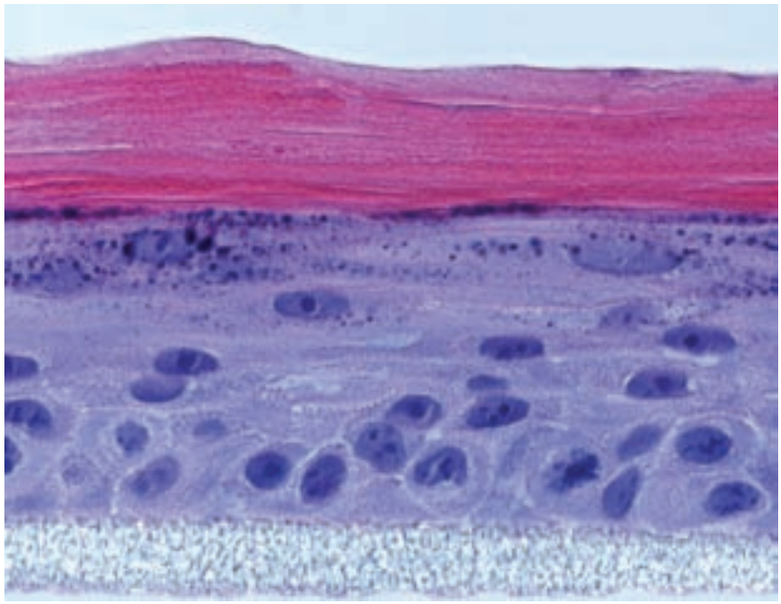

Figure 12: A fully differentiated 3D epithelial model, established from a population of epidermal keratinocyte progenitor cells isolate in CELLnTEC Progenitor Cell Targeted medium and differentiated in CELLnTEC optimised 3D medium

microlitres/day. An innovative microfabricated perfusion barrier promotes uniform nutrient transport into the gel chamber. They key advantages of the 3D:M design are: 1) enabling 3D perfusion screening; 2) high quality cell imaging in a 3D environment; and 3) reduction of cell/gel usage by 10 times. CellASIC has also developed variations of the 3D microfluidic culture concept to address applications requiring: larger gel chambers, automated solution exchange, maintaining long term spatial gradients, and in-chamber immunostaining (**Figure 11**).

CELLnTEC Advanced Cell Systems (www.cellntec.com) applies the latest developments in adult stem cell signalling to formulate speciality cell culture media unique to the epithelial market. CELLnTEC began as a CRO developing 3D vaginal and bladder models. This led to a number of epithelial culture media, and primary cells isolated in these media especially suitable to 3D cell culture. CELLnTEC has optimised a skin medium that supports the conflicting requirements of 3D *in vitro* modelling. 3D models place very specific demands on the cell culture medium. Specifically, the medium must encourage cells to reach terminal differentiation and stratify, while in parallel maintaining the population of proliferative cells necessary in the basal layer to establish a fully stratified model. These conflicting requirements are poorly addressed by conventional media, which were developed solely on the need for isolation efficiency and proliferation, without consideration of these parallel requirements of a 3D model. In combination with primary human keratinocytes isolated in CELLnTEC's Progenitor Cell Targeted media, this 3D medium has been found to establish 3D epidermal models with accurate representation of the *in vivo* structure, marker expression and lipid profile and to maintain them for an extended period. CELLnTEC also has a thriving CRO business testing compounds on these skin models. Additionally, outside laboratories are using its cells and media for 3D Airway models, corneal transplantation research and 3D co-culture experiments. CELLnTEC has recently invested in new facilities and staff to extend its offering of *in vitro* 3D models both via CRO and packaged products (**Figure 12**).

The 'Cell Cycle' is a partnership formed between the **European Collection of Cell Cultures** (ECACC) (www.hpacultures.org.uk), **Sigma Aldrich** (www.sigmaaldrich.com), **Corning** (www.corning.com) and **XCellR8** (www.x-cellr8.com). This partnership fully recognises that proliferation and differentiation of cells in culture has become increasingly important for basic research, drug discovery, tissue engineering and regenerative medicine. The way in which cells proliferate and differentiate is defined by the unique microenvironments required by the different cell types in a multicellular organism. Several factors influence the cells' proliferative and differentiation status and these fall into two broad categories: soluble cues (growth factors, metabolites, dissolved gases) and insoluble, physical cues (the composition, architecture,

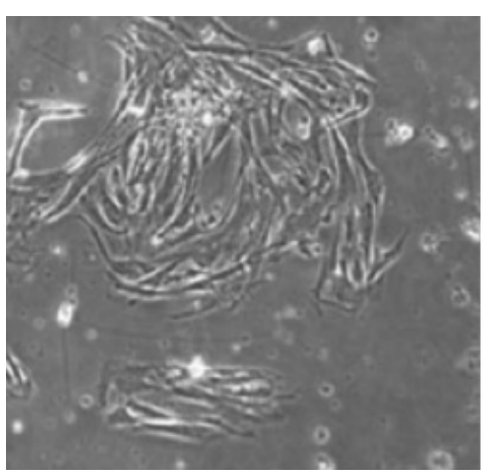

Figure 13: Human mesenchymal stem cells grown by ECACC for five days on the surface of a HyStem hydrogel with non-covalently incorporated collagen I

and elasticity of the extracellular matrix (ECM) and cell-cell interactions). The incorporation of these cues for *in vitro* cultivation of cells is crucial for recreating a cell's natural niche. For example, in myogenic induction, it has been found that the MyoD1 muscle marker protein will only be fully expressed by Murine muscle cell cultures when the hydrogel matrix stiffness matches that of muscle (11kPa). Hydrogel systems such as Glycosan Biosystem's HyStem™, allow researchers to design environments in which cells respond in a physiologically relevant manner. HyStem™ is a customisable synthetic hydrogel that can be used as an *in vivo* animal-free cellular delivery vehicle which is biocompatible, biodegradable and injectable. HyStem™ is optimal for culturing stem cells whose natural environments are rich in hyaluronic acid. The HyStem™ hydrogel scaffold closely mimics the rich, natural extracellular matrix environment, complete with hyaluronic acid and collagen fibrils, into which appropriate growth factors, attachment factors, and proteins can be incorporated selectively by the researcher. The Cell Cycle partnership is working to deliver complete cell culture solutions for researchers (**Figure 13**).

As the biomedical research community continues to make extraordinary strides in the prevention, diagnosis, and treatment of debilitating diseases, it has become more critical that reliable, efficient, affordable, and relevant techniques for the culture of cells, particularly stem cells, become available. **Global Cell Solutions (GCS)** (www.globalcellsolutions.com) along with **Hamilton Company** (www.hamiltoncompany.com) have developed the ideal cell culture system that allows for 3D stem cell cultures at the benchtop. This cell culture solution is a magnetic, pipettable and application-friendly microcarrier called the GEM™. The GEM™ consists of a magnetic core, an optically clear and non-autofluorescent alginate microsphere, and a thin molecular layer of basement biomimetic coating. Cells are loaded on to the GEM™ where the coating allows them to divide and expand. GCS offers a variety of kits to researchers, including one with the pre-coated and ready-to-use laminin and basement membrane GEM™ microcarriers to promote the expansion and maintenance of most adherent stem cells feeder-free. Stem cells can now be magnetically manipulated opening the door for co-cultures and novel experiments. The cells can be sampled, transferred, monitored for pluripotency, assayed, imaged and cryopreserved directly on the microcarrier. Furthermore, they never have to come near trypsin during these applications. The magnetic GEM™ can be gently levitated and dispersed in the growth media while also enabling full automation of the cell culture process. The alginate core of the GEM™ shares properties with hyaluronic acid which has been shown to affect differentiation. Thus, the GEM™ makes 3D stem cell culture convenient, reliable and efficient (**Figure 14**).

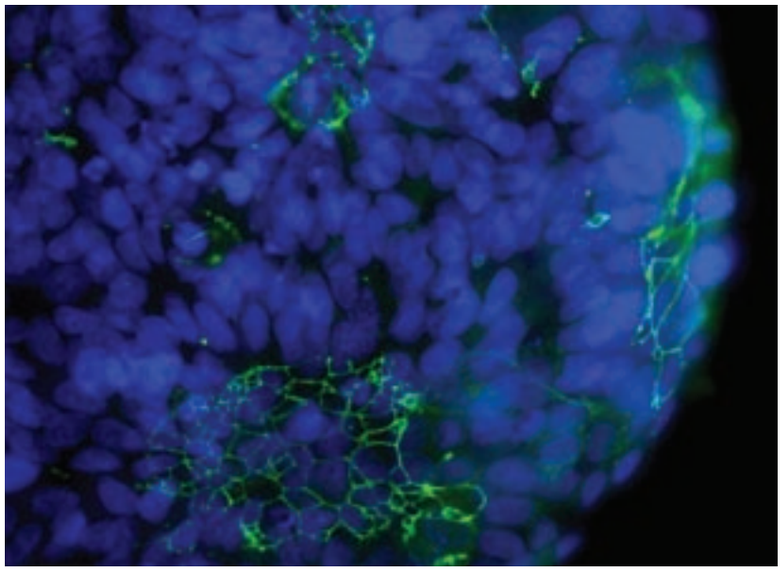

Figure 14: Human neural stem cells begin directed differentiation on the Global Cell Solutions GEM *(image courtesy of Life & Brain)*

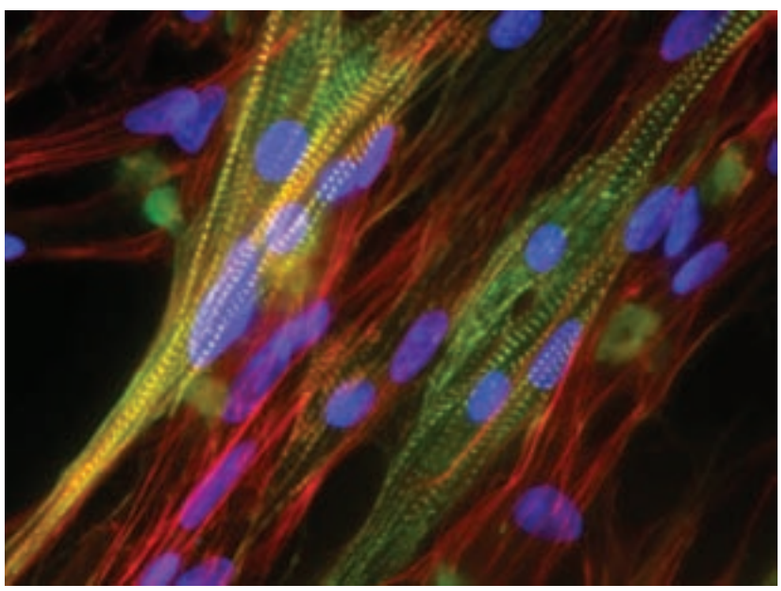

Figure 15: Chicken embryonic cardiomyocytes (striations) and fibroblasts (no striations) cultured with Glycosan BioSystems HyStem hydrogel. Blue = Nuclei, Red = Actin and Green = Alpha-Actinin

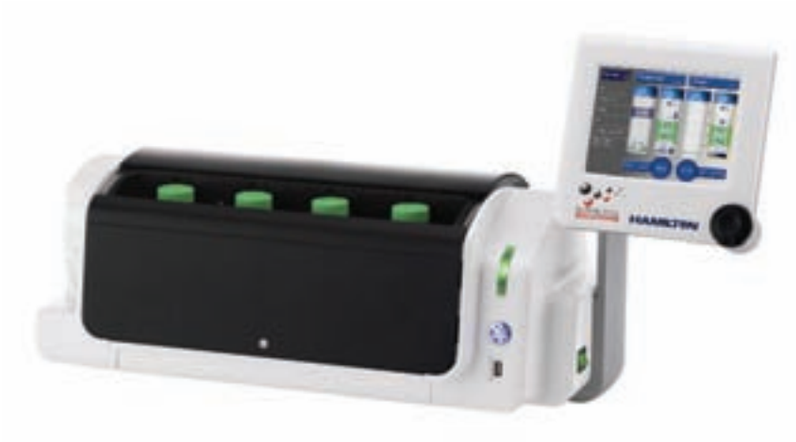

Figure 16: Hamilton Company's BioLevitator™ 3D cell culture system

Glycosan BioSystems (www.glyconsan.com) manufactures and sells Extracel™ and HyStem™ hyaluronic acid-based hydrogels for 3D and stem cell culture. As 3D cell culture increases in popularity, a key need for researchers is to gently and rapidly recover encapsulated cells for either nucleic acid or protein extraction. Glycosan has recently launched Extracel-SS and HyStem-CSS hydrogels which dissolve within two hours. The key improvement is the novel crosslinker, PEGSSDA, which has an internal disulfide bond which breaks upon exposure to reducing agents (**Figure 15**).

Hamilton Company (www.hamiltoncompany.com) and **Global Cell Solutions** (www.globalcellsolutions.com) have joined forces to develop the BioLevitator™, a unique 3D cell culture system. The instrument is a self-contained incubator and bioreactor with temperature and CO_2 control. The BioLevitator is used to culture adherent cell types on a unique 3D magnetic substrate called the Global Eukaryotic Microcarrier or GEM™, developed by Global Cell Solutions. The GEM is composed of an alginate core with covalently bound proteins, which allows the cells to grow on the 3D curved surface. Multiple protein coatings are available to support established, cancer, primary, and stem cell lines. Cells cultured on the GEM, including primary and stem cells, are healthier and have shown an improved *in vivo* phenotype. The GEM is non-autofluorescent and optically clear making cell-based assays much more user friendly. Cells can be easily assayed via fluorescence, luminescence, or absorbance detection methods. Additional downstream applications, including electroporation and cryopreservation, have shown enhanced efficiency when the cells are attached to the GEM. The cell culture parameters of the BioLevitator are directly scalable to the 3D-CellHOST. The 3D-CellHOST integrates up to four BioLevitators on Hamilton's MICROLAB STAR automated liquid handling platform and has the ability to produce two billion cells per week. The automated platform eliminates user error and variation between cell lines and users. The latest advancement in the BioLevitator technology is the addition of a pH reader, for online monitoring of the quality of the cell culture medium and automated medium change (**Figure 16**)

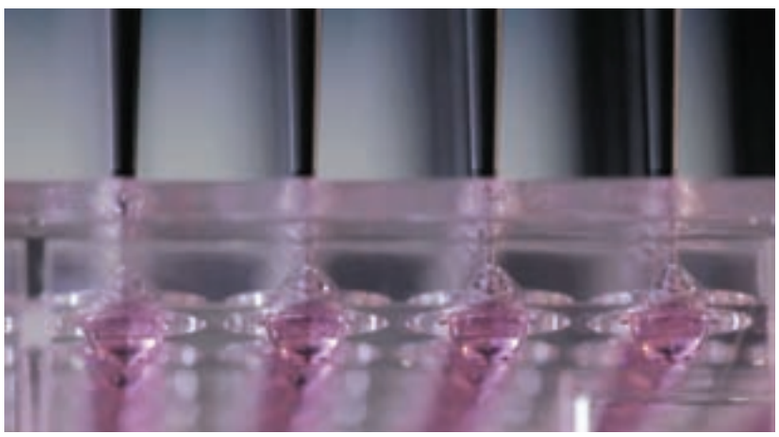

Figure 17: Automated generation of hanging drops to produce microtissues using the InSphero GravityPlus technology

Microscale organotypic cell-culture technologies are currently shifting from the academic environment to an industry setting enabling better model systems to test for drug efficacy and toxicology *in vitro*. Over the past few years, the hanging drop technology has shown its versatility to recreate embryonic, tumour and primary tissues *in vitro*. However, the conventional hanging drop technology – placing drops of cell suspension on a surface which is cultivated upside down – did not allow for high-throughput production of microtissues. The GravityPlus technology from **InSphero** (www.insphero.com) allows for generating hanging drops as well as medium exchange from the top enabled by a special well-design where the inlet and the culture compartment are connected via a vertical microchannel. The capillary and cohesion forces lead to highly stabilised hanging drops which can be handled either manually or in an automated fashion. This system enables InSphero to produce microtissues with a number of substantial advantages: a) low cell numbers required for one tissue depending on the required size (100-25,000); b) no adverse effects of artificial materials on biochemical assays; c) standardised tissue size and one tissue per drop enables

precise microscopic analysis and excellent cell number-size correlation; and d) the possibility to increase the complexity from homotypic to heterotypic tissue models. These tissues are provided off-the-shelf or custom-made in 96-well-compatible plates to allow for a straightforward upgrade from 2D to 3D at the user lab using existing instruments and assay procedures (**Figure 17**).

Kuraray (www.kuraray.co.jp) is developing a new cell culture plate called the Micro-Space Cell Culture plate by utilising its micro-fabrication technology. The plate has a number of micrometer size compartments regularly arrayed on its surface which provide cells 'micro-space' to form 3D structure. The plate has several advantageous features: it conforms to the standard microplate footprint enabling simple handling; it has good observability; and there is uniformity in the size or shape of the microstructure. No special techniques, including gel formation, are required. The bottom of the plate is a thin film made of transparent material, polystyrene, so that it is suitable for microscopic observation for fluorescent immunoassay. The size of the cell aggregates can be controlled by the size of the micro-pattern used. The shape of the micro-pattern can be designed as desired. It is believed that an optimum micro-pattern varies depending on cell type. For instance, primary hepatocytes form a spheroid-like structure in a square compartment (eg surface which width x 100µ depth) (see **Figure 18**). Hepatocyte-specific functions, for example albumin secretion and some of cytochrome P450s activity, were well preserved on the Micro-Space Cell Culture plate compared to those on a conventional flat plate by several-fold as measured by mRNA expression. On the other hand, cardiomyocytes form cell networking on another pattern providing synchronous contraction, and mammary epithelial cells present acinus-like structure on other pattern showing similar morphology to that seen *in vivo*. The Micro-Space Cell Culture plate is currently nearing commercialisation. Kuraray has prepared 24-well and 96-well sample plates for collaborators in Academia and Pharma, but other formats 6-, 12- and 384-well will be made if Kuraray confirms demand. Kuraray believes the plate is applicable for HTS due to the simple handling and good observable feature.

QGel (www.qgelbio.com) recently commercialised QGel™ MT 3D Matrix (QGel™), a synthetic hydrogel supported by more than 10 years of scientific experimentation and publications (**Figure 19**). Among many of its applications, QGel™ can be used as a matrix for 3D cell culture and is particularly interesting for studies in regenerative medicine, cancer research and drug screening. The synthetic biological and biochemical components used to bioengineer QGel™ extracellular matrix (ECM) are what make QGel™ unique compared to currently available products. Protease-sensitive sites and cell-adhesion ligands, such as RGD (peptide containing the amino acid sequence Arginine-Glycine-Aspartic Acid), are key components of QGel™ that mimic essential features of natural ECM (**Figure 19**). Unlike other synthetic products, QGel™ degrades like natural ECM, ie by cell-secreted and activated proteases, such as MMP (matrix metalloproteinase). QGel™ components

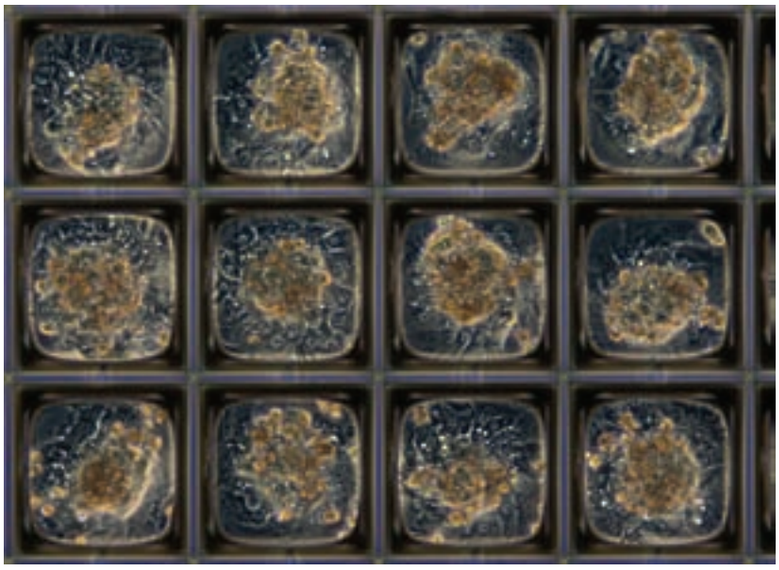

Figure 18: Microscopic observation of primary hepatocytes on Kuraray Micro-Space Cell Culture plate

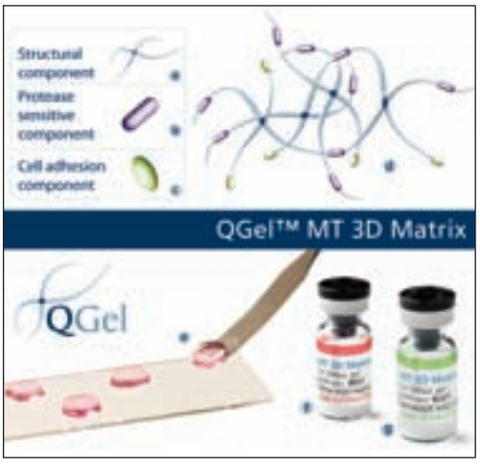

Figure 19: QGel™ is synthetic gel that consists of a polymer (a) structural component, (b) protease sensitive sites, and (c) cell-adhesion ligands. These three components are pre-mixed as a powder in a single vial. After solving the powder with QGel™ Buffer (not shown) a (d) 3D hydrogel structure forms that mimics key features of the natural extracellular matrix. Gelation occurs in 5-10 minutes, which allows you to encapsulate cells and shape the gel as you wish, such as in the form of a (e) 3D disc. QGel™ matrix products are available as (f) non-degradable and (g) degradable gels (with and without incorporated RGD-peptide) delivered sterile in ready-to-use vials for 0.5mL of gelated matrix

Figure 20: Tissue Equivalent Culture in the RealBio™ D4 Culture System

that define the biological and biochemical characteristics of its matrix are like building-blocks that allow for QGel to be bioengineered on a molecular level. This building-block modularity gives QGel™ advantages over natural 'gold standard' matrices, where 'what you get' depends on the origin of the organism from which it was derived. The ability to synthesise and bioengineer QGel™ offers scientists an exclusive possibility to adapt these biomimetic 3D cell culture models to specific cell types and to investigate different aspects of cell-matrix interaction in 3D. QGel™ matrix products are available in sterile, ready-to-use vials manufactured under strict GMP conditions.

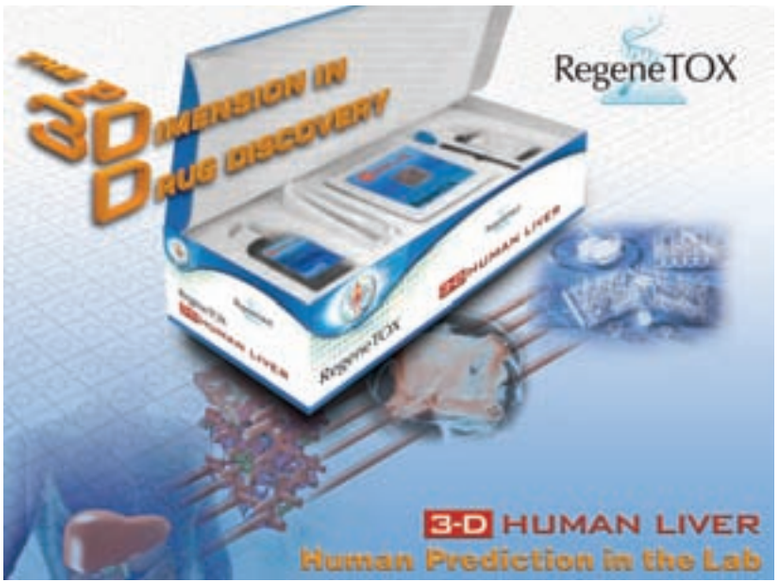

Figure 21: RegeneMed Liver3 and RegeneTOX product line for safety and efficacy assessment of new chemical entities

RealBio™ Technology (www.realbiotechnology.com) has introduced the D4 Culture System, a revolutionary tool for growing cells and tissues in a setting that closely mimics the *in vivo* environment. The RealBio™ D4 Culture System represents an exponential improvement over current 2D and 3D cell culture technologies because it supports mixed-cell populations in varied microenvironments throughout an open scaffold. As a result, tissues and cells cultured in the D4 Culture System arrange as *in vivo* and demonstrate natural function. In addition, cells cultured in the D4 system exhibit migration throughout and out of the tissue mass, just as cells naturally migrate *in vivo*. The D4 Culture System also provides researchers with unprecedented control of the culture environment, including control of nutrient and gas gradients across the cultured tissue mass, by decoupling the supply of nutrient media and metabolic gasses. The D4 Culture System was originally developed with a $5 million grant from the US military and perfected within the RealBio™ Technology laboratory in Kalamazoo, Michigan. The patented D4 System is a valuable tool for studying various structured tissues (including polar tissues) and has demonstrated the ability to support the growth and maintenance of human bone marrow for longer than one year with daily harvests of a mixed-cell population that includes early progenitor stem cells. Mixed-cell populations harvested from long-term cultures maintained in the D4 system exhibit similar CD marker profiles to the initial seed material thereby demonstrating that the RealBio™ D4 Culture System minimises the selective pressures observed in other culture systems (**Figure 20**).

RegeneMed (www.regenemed.com) provides 3D human and animal tissue cultures in multiwell plates and specialised bioreactors to serve as more physiologically relevant replacements to current industry-standard animal and cell-based tests that are often not predictive of the human response to toxic compounds. Liver3, 3D liver co-culture, sustains tissue function for months, providing off-the-shelf available, reproducible, reusable, prediction of metabolism, toxicity and efficacy measures for new drug candidates, chemical entities and consumer products. The long-term tissue-specific function enables pre-characterised tissues from fresh and cryopreserved hepatocytes for *in vitro* assessment of previously unattainable endpoints such as bioavailability, drug-drug interactions and chronic

toxicity. The RegeneTOX product line provides tissues, customised assays and Contract Testing Services for safety and efficacy assessment of new chemical entities. Included are 2D hepatocytes and 3D liver co-culture from the same liver donor and sibling animal dosing for *in vitro* 2D to 3D to *in vivo* comparisons. Skin3 3D full- and partial-thickness skin models (based on the Skin2 product line pulled from the market in 1996 and expanded to include immune competent models to address sensitisation, inflammation, etc) will be re-launched to address the growing animal alternative market and regulations. Stem cell-derived 3D tissues, including liver, cardiomyocyte and neuronal, for patient-specific and genomic diversity research are in development. The mission of the company is to provide tissues and organs to address medical need regardless of disease progression state; from miniaturised tissue for high throughput safety and efficacy assessment, to chemical/biological warfare biosensors, molecular diagnostics, personalised medicines, medical devices and tissue implants (**Figure 21**).

Reinnervate (www.reinnervate.com) has researched and developed Alvetex™, a unique polystyrene scaffold designed as a platform technology to enable routine 3D cell culture. The company is now focused heavily on preparing its commercial facilities to support quality controlled procedures for the scaled manufacture and production of Alvetex™, due for formal product launch in various formats later this year. Its product quality testing levels exceed the various standards required for a consistently high performing cell culture product. This is essential for the global distribution of Alvetex™ to the industrial sector. Its development work includes the manufacture of various devices designed to present the scaffold, to enable optimal function and versatility. For example, well inserts, well clips and large reservoir vessels are all important ancillary components to maximise the benefits of 3D cell culture using Alvetex™. Reinnervate's technology is adaptable to scaled manufacture and it has overcome various challenges to deliver a quality 3D cell culture product. While its product is designed as a generic solution to 3D cell culture, scientists at Reinnervate are also developing specific applications for Alvetex™, notably: an artificial 3D skin construct for *in vitro* testing of cosmetics and topical drugs; a liver toxicity assay using 3D cultured hepatocytes; a 3D platform for enhanced cell differentiation by various stem cell types; and a cell invasion model to evaluate cell migration in 3D to assess various anti-cancer compounds. Alvetex™-enabled 3D cell culture will represent the core technology behind each of these applications for which there is significant market demand (**Figure 22**).

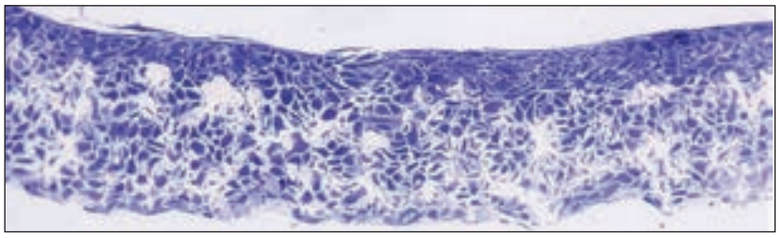

Figure 22: Reinnervate's Alvetex™ is supplied as a 200 micron thick membrane that enables cultured cells to form a 3D structure. This sample has been fixed, embedded, transverse sectioned, and cells stained with Toluidine Blue. Alvetex™ is seen in white

Expansion of hematopoietic stem cells for bone marrow transplantation using conventional 2D culture systems with exogenous growth factors has met with little success. Recent research has shown that the stem cell microenvironment (stem cell niche) plays a key role in the maintenance and expansion of stem cells. **Synthecon** (www.synthecon.com) has developed, with funding from the National Institutes of Health, a 3D, bio-artificial stem cell niche in its Rotary Cell Culture System (RCCS™) to address this problem. The bio-artificial niche consists of disk-shaped porous scaffolds, bone marrow stromal cells and umbilical cord

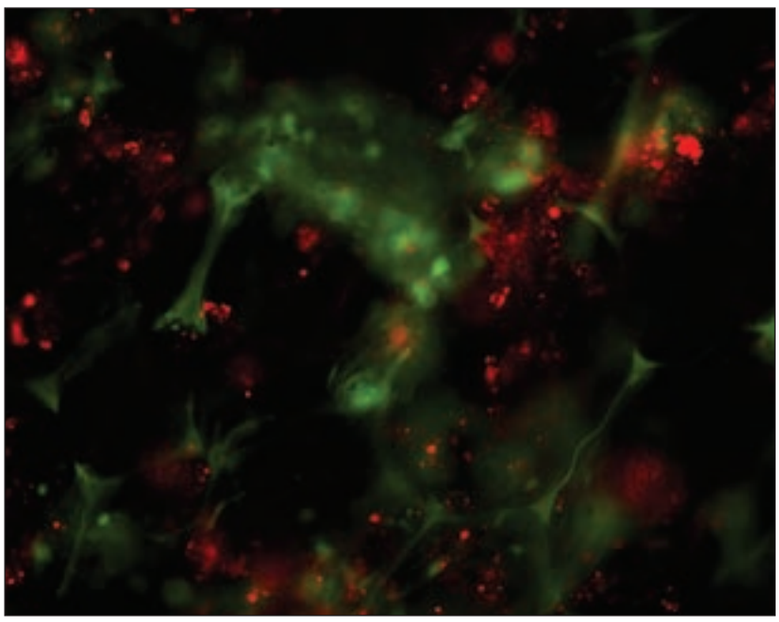

Figure 23: Bio-artificial niche assembled in the Rotary Cell Culture System. Bone marrow mesenchymal cells expressing GFP were grown on a porous 3D scaffold. Umbilical cord blood stem cells stained with DiIC18(5)-DS were added and allowed to home to the scaffold

Figure 24: The RAFT (Real Architecture for 3D Tissue) system from The Automation Partnership forms tissues in less than an hour by controlling compression and incubation of collagen-based gels seeded with cells. The system, which uses a SBS standard 24-well plate format can be filled manually and will fit into a standard laminar flow hood to prevent contamination of tissue constructs

blood stem cells. The bone marrow stromal cells (MSCs) are first seeded with the scaffolds in the RCCS and allowed to proliferate to confluence. The umbilical cord stem cells are subsequently injected into the bioreactor and induced to home to the scaffolds by chemoattractive signalling from the MSCs. Stem cells will be detached from the scaffolds by mobilising agents currently used *in vivo*. Synthecon has designed a modification of its standard RCCS that will allow harvesting of the stem cells without disturbing the bio-artificial niche. The goal of this project is to mimic the *in vivo* production of hematopoietic stem cells in a scalable bioreactor system that can reliably expand these cells for therapeutic applications (**Figure 23**).

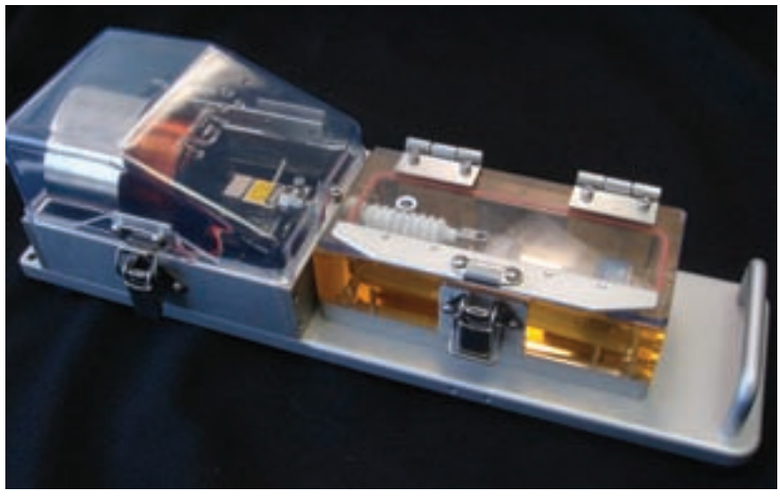

Figure 25: Tissue Growth Technologies' DermiGen™ Mechanical Stimulation Bioreactor System

RAFT (Real Architecture for 3D Tissue) is a new system for scientists to create consistent, well defined 3D tissues in a convenient, simple-to-use format. It has been developed by **The Automation Partnership** (**TAP**) (www.automationpartnership.com) in collaboration with leading tissue engineering academics and uses a novel, patented technology for making 3D collagen tissue constructs rapidly (<1 hour), simply and reproducibly. Cells and neutralised collagen are mixed, pipetted into a special 24-well plate and incubated. Gentle, controlled compression is applied to the cell seeded hydrogels (by absorbent plungers) which removes some liquid, increasing the cell and collagen concentration 100-fold. The construct surface can be embossed with micro scale topology, and could be used for example, to mimic the *in vivo* stem cell microenvironment, and then culture a different cell type on the surface. RAFT allows complex multilayer tissues to be formed – with different cell types in each layer – and cells to be co-cultured in a well controlled way. The resulting biomimetic tissues, made from fibrillar collagen (the main component of extracellular matrix), are strong, transparent and 50-100µm thick. TAP has developed a workstation and consumables to automate and scale up this 3D tissue production process, enabling up to 24 tissues to be made in parallel. Tissues are made either in the wells of a 24-well plate; on permeable membrane inserts for barrier assays or cultured at an air/liquid interface, for example to form stratified epithelia. The tissue remains in the same well from its creation until the end of the experiment and can be analysed using standard techniques. TAP will be unveiling RAFT at the Tissue Engineering and Regenerative Medicine International Society (TERMIS) Conference, on June 13-17, 2010 in Galway, Ireland (**Figure 24**).

The DermiGen™ Mechanical Bioreactor, manufactured by **Tissue Growth Technologies** (www.tissue-growth.com), imparts static and oscillatory strain to a skin-like sample in a unique air/media interface. The device may be used to stimulate the growth of tissue engineered skin or to act as a test bed for drug and cosmetic development. Featuring a linear motor driven mechanical stimulator, the DermiGen applies mechanical strain using operator defined parameters. The bioreactor's unique chamber design provides nutrient media perfusion

along the underside of the specimen while simultaneously exposing the topside to a gaseous environment. The chamber accommodates sample dimensions of 70mm length, 20mm width and 0.1-2mm thickness. The company's GrowthWorks Software and Controller runs on a Windows® laptop and can operate up to four mechanical stimulators. The entire system is designed to fit within a standard laboratory incubator and the chambers can be autoclaved. Founded in 1990 with the charter of becoming a world-class provider of instruments for the biomedical community, Tissue Growth Technologies has introduced bioreactors for tendons, ligaments, cartilage, bone, blood vessels, heart valves and other 3D tissue types (**Figure 25**).

Discussion

Table 1 attempts to summarise the main features of the 3D cell culture offerings that vendors submitted for inclusion in this review. Although this list does not represent all available offerings, it is fairly representative of what is currently commercially obtainable to support 3D cell culture.

The majority of these developments utilise some sort of biomimetic scaffold (eg hydrogel or collagen) (3D Biotek, BellBrook Labs, CellASIC, ECACC, Global Cell Solutions, Glycosan, Hamilton, QGel, The Automation Partnership). It is generally accepted that biomimetic scaffolds offer a better prospect to culture specific cell types and to investigate different aspects of the cell-matrix interaction in 3D. Several trends are evident in this respect to biomimetic scaffolds: 1) the move to using synthetically derived materials to minimise the previously poor reproducibility between batches and the resulting lack of consistency between cultures (especially primary cells) (QGel); 2) the ability to design scaffold environments so cells respond in a physiologically relevant manner, eg stem cells are thought to do better in gels rich in hyaluronic acid (ECACC, Global Cell Solutions, Glycosan); and 3) the development of biodegradable scaffolds, particularly to support applications in tissue engineering and stem cell research (3D Biotek, Glycosan). As 3D cell culture emerges in popularity, a key need for researchers in these areas is to gently and rapidly recover encapsulated cells from scaffolds for nucleic acid and protein extraction.

Apart from the biomimetic scaffolds there are what we have called in **Table 1** 3D structural scaffolds. Most of these are made from the same material as 2D plate surfaces (ie polystyrene), but offer additional 3D microstructure directly on the 2D surface, or are produced separately and mounted on to a 2D surface or supplied as an insert to a 2D culture vessel to create a 3D structurally matrix (3D Biotek, Kuraray, Reinnervate, Synthecon). All provide cells with a microspace to form 3D structure and demonstrate enhanced functional activity compared to cells grown under identical conditions on 2D culture plastic. Being for the most part inert polystyrene structural scaffolds are non-biodegradable, but are compatible with most routine (easy) 2D assays and have optically clarity allowing researchers to monitor 3D cell growth by simply using an inverted light microscope. The uniformity of the 3D matrix does however vary; where they are micro-fabricated directly on to the 2D surface they are usually highly uniform repetitive patterns. Where they are fabricated as an emulsion-templated polystyrene scaffold which is subsequently mounted on to a 2D surface the architecture is slightly less regular, but is highly porous and consists of voids linked to one another by pores.

We also report on several microfluidic devices (BellBrook Labs, CellAASIC) that have moulded microchannels contained within a microplate format for ease of handling and automation. These offerings incorporate a biomimetic scaffold (gel) and are structured to enable fluidic flow or long term perfusion of cells and to facilitate high quality cell imaging in a 3D environment. Currently they are optimised to quite specific 3D applications (eg invasion assay profiling) or to support specific tumour cell models.

Only three platforms that directly support the automation of 3D cell culture and/or tissue creation were reported in the vendor's snapshots. Awareness of these platforms was not particularly high among HTStec's survey participants, although since all are relatively new approaches, this is not surprising. The development of microcarriers (magnetic, spherical, pipettable substrates) (Global Cell Solutions) and the associated relatively small scale automation of 3D cell culture on these microcarriers (Hamilton Company) represents a major advance in manipulating, culturing and even cryopreservation of cells, however the suitability of these microcarriers to a significant proportion of cell and tumour lines used for screening is in doubt[1]. Nevertheless the simplification to the work flow of cell-based screening made possible by such a format has triggered additional investigations, particularly with respect to undertaking bioassays directly on microcarriers (Biotek). An alternative approach that has yielded some success in recreating 3D embryonic, tumour and primary tissues *in vitro* is hanging drop scaffold-free culture. The advent of

Table 1: Summary of the features of the 3D cell culture offerings discussed in this article

3D Vendor Company	Microplate Based	Device Type	Biomimetic Scaffolds	3D Structural Scaffolds	3D Specific Culture Media	Facilitates Automation	Tissue Creation
3D Biotek	√	Well insert	√	√			√
BellBrook Labs	√	Microfluidic plate	√			√	
BioTek						√	
CellASIC	√	Microfluidic plate	√				
CELLnTEC Advanced Cell Systems					√		√
ECACC/HPA Culture Collection			√		√		√
Global Cell Solutions			√			√	√
Glycosan			√				√
Hamilton			√			√	√
InSphero	√	Hanging drop				√	√
Kuraray	√	Wells		√		√	
QGel			√				√
RealBio Technology		Flow chamber		√			√
RegeneMed	√	Wells, well inserts					√
Reinnervate		Well inserts		√			√
Synthecon				√			
The Automation Partnership	√	Wells, well inserts	√			√	√
Tissue Growth Technologies							√

GravityPlus technology (InSphero) provides a means for high-throughput production of microtissues and for the automation of media exchange from hanging drops. The RAFT (Real Architecture for 3D Tissue) system (TAP) is a convenient, simple-to-use semi-automated way of bringing greater scale-up and consistency to generating tissue constructs by controlling compression and incubation of collagen-based gels seeded with cells. It remains to be seen what impact these early attempts at automation will have on 3D cell culture and tissue generation, and whether any will be adopted to a large extent.

The need for more physiologically relevant replacements to current industry-standard animal and cell-based tests of efficacy and *in vitro* toxicology was highlighted by several vendors and 3D model systems of liver (hepatocytes), skin, cancer invasion, vaginal, bladder and airways are beginning to be developed to meet this need (BellBrooks Labs, CellASIC, CELLnTEC, InSphero, RegeneMed, Reinnervate, TAP). We also report on a device that may be used to stimulate the growth of tissue engineered skin or to act as a test bed for drug and cosmetic development (Tissue Growth Technologies) and a culture system with a flow chamber that utilises a 3D matrix to support culture development and incorporation of normal tissue organisation (RealBio).

Stem cells are the prime application focus of 3D cell culture. This is partly because the *in vivo* microenvironment is believed to play a key role in

	Tissue Creation	3D Model Development	Stem Cell Focus	Other Features/ Applications:
	√			
		√		HCS imaging
				Plate reading
		√		HCS imaging
	√	√		Services
	√		√	Cell supply
	√		√	Cell supply
	√		√	
	√			Bioreactor, Cell supply
	√	√		Cell supply
		√		HCS imaging
	√			
	√	√	√	Prolonged culture, Cell harvesting
	√	√	√	Services
	√	√	√	
		√	√	Bioreactor
	√	√	√	
	√			Bioreactor

the maintenance and expansion of stem cells and 3D is perceived as a way of manipulating this microenvironment. Also conditioned media derived from mesenchymal stem cells grown in 3D matrixes has instructive effects on the differentiation of some stem cells. Quite a number vendors highlighted in this review are developing and optimising their 3D offering to further stem cell research (ECACC, Global Cell Solutions, Glycosan, RegeneMed, Reinnervate, RealBio, Synthecon, The Automation Partnership).

Reading the vendor snapshots one might conclude that 3D cell culture was a done deal and tissue generation is readily achievable, however HTStec's survey uncovered many problems and unmet needs, for example:

- Poor reproducibility between batches of biomimetic scaffolds.
- 3D matrices have too many components and creation of constructs is difficult and laborious.
- Limited ability to scale up or down a single 3D format.
- Post culturing processing/cell extraction from matrix difficult to handle.
- Virtual lack of proven automated solutions.
- All methods need higher throughput.
- Lack of methodology directly applicable to screening and bioprocessing.
- Wider applicability needed, today everything depends upon or is limited to the specific model.
- Lack of consistency between 3D cultures (especially primary and stem cells).
- Greater flexibility to accommodate the many different cell-lines and types.
- Better methods (readouts) for characterising cells cultured in these geometries.
- Better visualisation, wider applicability to HCS and video imaging, better image analysis tools.
- Room for improvement with more physiological substrates.
- Limited stability in long term experiments.

In conclusion, the state-of-the-art seems to some way off from providing fully validated or robust 3D culture solutions and the field is clearly open to major improvements at this point in time. DDW

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Reference

1 3D Cell Culture Trends 2010 Report, published by HTStec Limited, Cambridge, UK, February 2010.