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Three-Dimensional Cell Cultures in Toxicology

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Abstract

Toxicity testing with animals is expensive, ethically controversial, and not always predictive of the human response. Cell-based assays are regarded as an alternative. However, conventional two-dimensional cell cultures do not reproduce the tissue architecture *in vivo,* and do not forecast organ-specific toxicity. On the other hand, three-dimensional cultures emulate the biochemistry and mechanics of the microenvironment in tissues more closely. Therefore, they address the limitations of both animals and two-dimensional cultures, and provide more accurate data on the effects of short- and long-term exposure to toxicants. We provide an up-to-date overview on the use of three-dimensional cell cultures in toxicology. We anticipate that three-dimensional cultures will become invaluable to accomplish the 3R agenda (refinement, reduction, and replacement) for animal-based toxicity testing and will play a major role for the Registration, Evaluation and Authorisation of Chemicals in the European Union (REACH legislation).

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Abbreviations: 3R, Refinement, Reduction, and Replacement; REACH, Registration, Evaluation, and Authorization of Chemicals; ADME, Absorption, Distribution, Metabolism, Elimination; 3D, three-dimensional; 2D, two-dimensional; ECM, Extracellular Matrix; MDCK, Madin-Darby Canine Kidney; EHS, Engelbreth-Holm-Swarm; GOT, glutamic oxaloacetic transaminase; GPT, GPT, glutamic-pyruvic transaminase; LDH, lactate dehydrogenase; gamma-GT, gamma-glutamyl transferase; ISC, intestinal stem cells; ECVAM, European Center for the Validation of Alternative Methods; CYP, cytochrom P450; RWV, rotating well vessel; HCS, high content screening; ESEM, environmental scanning electron microscope; LSFM, light-sheet-based fluorescence microscope; SPIM, single plane illumination microscope; DSLM, digital scanned light-sheet microscope.

Introduction

The toxicity of a substance is usually tested using animals. The tests are time-consuming and expensive, as well as ethically controversial, and in general of limited reliability. A comprehensive survey of drug screening tests has shown that 57% of the toxicological data derived from experiments with rodents do not correlate with the results of human trials (Olson *et al*., 2000). In many cases, drugs have failed during clinical trials due to their adverse toxicity. Pre-clinical toxicity tests with multiple animal species are also poorly predictive (de Boo & Hendriksen, 2005; Knight, 2007a; Knight, 2007b). These facts motivate governments and authorities to support alternative methods. The replacement of animal models for testing drugs and chemical substances is one of the three Rs of the Replacement, Reduction, and Refinement (3R) agenda for the humane handling of laboratory animals (de Boo & Hendriksen, 2005). The 3R compliancy is required as a good laboratory animal practice. Important regulatory initiatives, such as the European REACH (Registration, Evaluation and Authorisation of Chemicals) (Foth & Hayes, 2008; Lilienblum *et al*., 2008), require the application of alternatives to animal-based tests. To date, several basic cytotoxicity and ADME-Tox (drug Absorption, Distribution, Metabolism, Elimination and Toxicity) tests rely on cell cultures (Hasspieler *et al*., 2006; Li, 2001; Lin *et al*., 2003; Rausch, 2006). However, conventional two-dimensional cell cultures fail to detect organ-specific toxicity (Mazzoleni, Di Lorenzo & Steimberg, 2009). The reason seems to be that the flat culture plastic substrates provide a non-physiological environment to the cell (Mazzoleni *et al*., 2009; Pampaloni, Reynaud & Stelzer, 2007). Plastic substrates are two-dimensional (2D) and fairly stiff. In contrast, real tissues have a three-dimensional (3D) geometry, gel-like (soft) stiffness, and a specific biochemistry determined by the proteins of the extra-cellular matrix (ECM). Examples of ECM proteins are collagen, laminin, and fibronectin. Although a 2D substrate can be coated with a thin layer of ECM proteins (usually collagen I or fibronectin) this does not seem to reproduce the complexity of the ECM *in vivo*. Due to the non-physiological microenvironment, cells mostly proliferate and de-differentiate in 2D cultures (Bhadriraju & Chen, 2002). Fibroblasts cultured in 2D have a flat shape strikingly dissimilar from the bipolar/stellate shape found in tissues (Beningo, Dembo & Wang, 2004; Rhee & Grinnell, 2007; Rhee *et al*., 2007). A comparison of the gene expression profile of melanoma cells showed that 173 genes differ between the same cells cultured in 3D and 2D (Birgersdotter, Sandberg & Ernberg, 2005). Most of the genes strongly up-regulated in 3D are chemokines, as well as laminin and c-Jun (Ghosh *et al*., 2005). Expression profiles of vascular smooth muscle cells showed evidence for ~100 genes, which are differently up-regulated in 2D compared to the situation in 3D (Li *et al*., 2003). Primary hepatocytes plated in 2D systems lose liver-specific functions after a few passages. The first function lost is the biosynthesis of the drug metabolizing enzymes, which are essential for testing toxicity (Gomez-Lechon *et al*., 1998; Pampaloni *et al*., 2007). On the other end, culturing hepatocytes in 3D collagen or purified basement membrane maintains liver-specific functions for several weeks. An extended liver-specific functionality is also obtained by aggregating hepatocytes into "spheroids" with a diameter of several hundreds of micrometers (Semino *et al*., 2003). Fibroblasts cultured in 3D collagen show a more typical *in vivo* phenotype (Cukierman *et al*., 2001). Madin-Darby canine kidney

(MDCK) cells generate kidney organoids (spherical hollow spheres called *cysts*) in 3D collagen (Figure 1a) (Montesano, 1986). This phenotype is not observed in 2D cultures of MDCK cells.

These selected examples show that establishing 3D cell-cell contacts and embedding cells in 3D gels that emulate the natural ECM reduces the gap between cell culture and real tissue. Here, we review the application of 3D cell cultures to toxicity screening. Properly standardized and validated toxicity assays based on 3D cultures will allow us to predict the effect of toxicants on humans with higher accuracy than both 2D cultures and animal models. 3D cultures could greatly improve the toxicity screening of industrial chemicals as well as eliminate the toxic substances at an early stage of the drug discovery pipeline. The available 3D systems still reproduce at rudimental level the microstructure and the function of live tissues. Substantial effort is currently devoted to optimize 3D cell cultures for toxicity screening purposes. This requires the introduction of new synthetic ECM-like scaffolds, bioreactors, as well as advanced imaging technologies.

Methods for 3D cell culture

3D HYDROGEL-BASED CULTURES

The natural ECM in tissues consists of a tight network of fibrous proteins filled with glycosaminoglycan hydrogel (Lutolf & Hubbell, 2005). ECM hydrogels for 3D cell cultures mimic the biochemical and mechanical properties of tissues. Cells are confronted with a more physiological condition compared to 2D systems. Therefore, the relationship between cell function and tissue architecture can be isolated and addressed. ECM hydrogels for 3D cell culture are either of animal origin or synthetic. The most often employed hydrogels in a 3D cell culture are collagen type I and basement membrane extract, both of animal origin.

COLLAGEN TYPE I

Collagen I is the prevalent ECM component of the connective tissue (stroma). Collagen is extracted from animal tendons and is commercially available as an acidic solution. A 3D collagen fibrillar hydrogel can be easily reconstituted *in vitro* by neutralizing the solution. A highly ordered fibrillar architecture, similar to the one *in vivo*, can obtained by controlling the collagen concentration and sonicating the solution before gelation (Bessea *et al*., 2002). Collagen hydrogels, sponges, microspheres, and membranes can be employed for 3D cultures (Chevallay & Herbage, 2000).

RECONSTITUTED BASAL MEMBRANE

The basement membrane is a fibrous sheet underlying the epithelia. It is composed of laminin 1, collagen III-IV, and heparin sulphate proteoglycans. The basement membrane hydrogel (commercial name "Matrigel" from BD Biosciences) is derived from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma (Kleinman & Martin, 1989) and can be reconstituted as a 3D gel under physiological pH and temperature. 3D

cell cultures in Matrigel and collagen I have been intensively and very successfully applied in breast cancer research (Bissell & Radisky, 2001; Cukierman *et al*., 2001; Petersen *et al*., 2001; Radisky, Hagios & Bissell, 2001), and to elucidate the steps leading to the establishment of epithelial cell polarity and epithelial morphogenesis (Montesano, 1986; Mostov, Su & ter Beest, 2003; O'Brien, Zegers & Mostov, 2002). These studies show very clearly that both morphology and gene expression adapt to the cell microenvironment.

The use of 3D cell cultures in hydrogels for toxicity screening requires standardized and biochemically well-defined matrices, which should suffer from minimal batch-to-batch variations. A significant improvement is the introduction of novel synthetic gels alternative to the "classical" extracted collagen I and to Matrigel, which is discussed in the last paragraph.

CELLULAR SPHEROIDS

Cellular spheroids are large (hundreds of micrometers in diameter) spheres composed of several hundreds to thousands of cells (*Figure 1b*). They form by aggregation of isolated cells. Cellular spheroids are among the first 3D cell culture models adopted in clinical pharmacology (Mueller-Klieser, 1997). An exogenous scaffold or matrix is not required to support the cells, since cell aggregation is spontaneous and facilitated by buoyancy or stirring. Spheroids can be obtained by the hanging drop technique (Kelm *et al*., 2003; Korff & Augustin, 1998; Timmins, Dietmair & Nielsen, 2004), by seeding cells on non-adhesive surfaces, such as 3D alginate porous scaffolds (Glicklis *et al*., 2000), or by employing rotating well vessel cultures (Bilodeau & Mantovani, 2006). Buoyancy is exploited in the "hanging-drop" method (*Figure 1b-1*). In this method, droplets of culture medium containing isolated cells are suspended from a Petri dish lid. After three to seven days of growth, large spheroids can be harvested (Kelm *et al*., 2003; Timmins *et al*., 2004). Alternatively, rotational stirrers can be employed for spheroids formation (Bilodeau & Mantovani, 2006; Moscona, 1961) (*Figure 1b-2*). The sedimentation of the cells within the vessel is offset by the rotating fluid, which keeps the cells continuously suspended in the culture medium. In a low-shear and low-turbulence regime, the rotating vessel bioreactors minimize the mechanical damage of cells and provide adequate nutrition and oxygenation.

SPHEROID TYPES

Many common cell lines can aggregate to spheroids, including MCF-10a (human mammary cell line), Caco-2 (intestinal cell line), and HepG2 (human liver cell line) (Kelm *et al*., 2003). Spheroids from human teratocarcinoma cell line Ntera2 (NT2) are a useful model system for biomedical studies and toxicity assays on the nervous system (Podrygajlo *et al*., 2009). Podrygajlo *et al*. have shown that the differentiation time of the to mature post-mitotic neurons can be drastically reduced from two to one month by aggregation in spheroids (Podrygajlo *et al*., 2009). Spheroids obtained from primary liver cells are particularly important for toxicity testing. Rat hepatocyte spheroids can be easily obtained by following standard procedures (Xu, Ma & Purcell, 2003a; Xu, Ma & Purcell, 2003b). During the transitional phase from

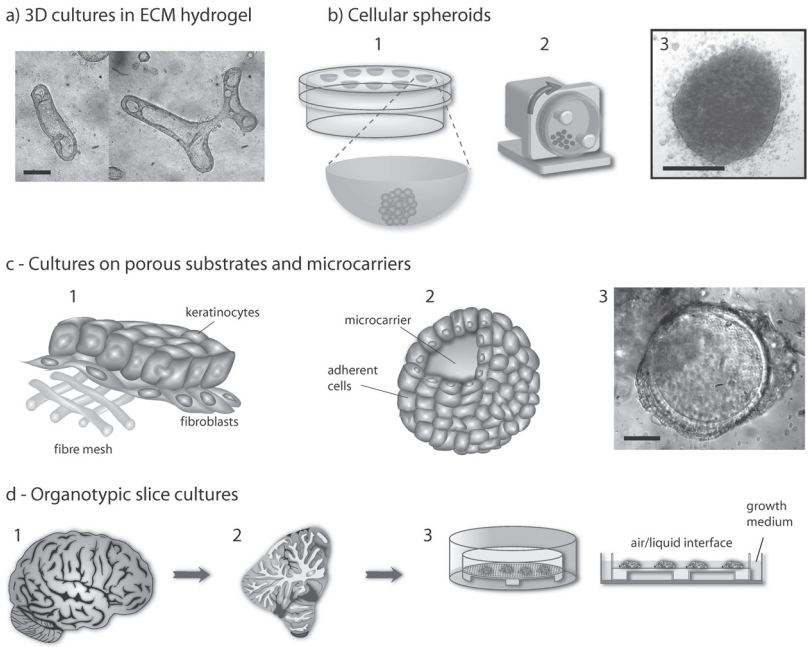

Figure 1. Examples of 3D cell cultures.

a. *3 D cultures in ECM hydrogels cultures.* An example is a 3D culture of MDCK cells (a kidney cell line) in hydrated collagen gel. The gel forms thick fibres, which support tissue-like cell growth. MDCK cells yields polarized epithelia resembling kidney tubules. The basal surface is in contact with the gel, and the apical side faces the fluid-filled internal cavity. The phase contrast micrograph shows MDCK tubes obtained by mechanically stretching the collagen fibers (scale bar 50 μm).

b. *Cellular spheroids*. Cells can re-establish mutual contacts and specific microenvironments that allow them to express a tissue-like phenotype by aggregating into large (several hundreds of micrometers) spheroids. These can be obtained at specific cellular concentrations within a "hanging drop" (1) or in rotating-wall vessels (2). In both methods, the cells cluster by gravity and then aggregate. (3) The phase contrast micrograph shows a spheroid obtained from the pancreas tumor cell line BxPC-3.

c. *Cultures on porous substrates and microcarriers*. (1) Culture on fibre mesh. For example, primary fibroblasts are cultured in Petri dishes. The cells are subsequently seeded onto a biodegradable fibre mesh. After several weeks in culture, keratinocytes, e.g. extracted from the foreskin, are placed onto the new dermal tissue and form an epidermal layer. (2-3) Microscaled materials beads derived from dextran, gelatine, glycosaminoglycans and other porous polymers can be used as a three-dimensional support for the culture of anchorage-dependent animal cell lines.

d. *Organotypic cultures*. Dissected organ slices, such as brain, are placed on porous substrates, supported by a metal grid (1, 2) and cultured at the air-growth medium interface (3).

isolated cells to mature spheroid (1-5 days) a drastic biochemical rearrangement has been observed in hepatocytes. Initially, glucose secretion and cellular activity of GOT and GPT increased (day 1-6). In contrast, LDH and γ-GT activity were undetectable during spheroid formation. Albumin secretion decreased rapidly during the first two days. Arginine uptake as well as urea and nitric oxide synthesis increased. The physiological liver-specific functions recovered with spheroid maturation (after day 6), and remained stable at least from day 6 until day 15. This nine day long time

window is recommended by the authors as the best suited interval to perform toxicity assays (Xu *et al*., 2003a; Xu *et al*., 2003b). In another study, the liver-like functionality of porcine hepatocytes has been extended to over 21 days by embedding them in 3D collagen (Lazar *et al*., 1995). Embryonic cells, such as retina cells (Layer *et al*., 2002), and adult multipotent neuronal stem cells (Jessberger, Clemenson & Gage, 2007; Wang *et al*., 2006) can also form spheroids (neuronal cells spheroids are termed "neurospheres"). Spheroids from embryonic retinal cells had been obtained by a static culture in a microscaffold cell array. Each microscaffold was a square with a lateral length of 300 μm. Each array contained 506 microcontainers. The cells produced mature spheroids within ten days (Rieke *et al*., 2008). This microarray format is promising for large-scale assays.

ADVANTAGES OF CELLULAR SPHEROIDS

Since the addition of exogenous extra-cellular scaffolds is not required, spheroid aggregation can be easily automated. Therefore, scaling-up cellular spheroids-based assays to high-throughput analysis is feasible (Friedrich *et al*., 2009; Ivascu & Kubbies, 2006; Kunz-Schughart *et al*., 2004; Zhang, Gelain & Zhao, 2005). Also, the relatively easy handling allows a precise patterning of the spheroids into 3D shapes. Jakab *et al*. positioned ten CHO cell spheroids within 3D collagen, patterning a circle. After ~5 days in culture, the single spheroids along the circle merged, forming a continuous tissue of toroidal shape. This work shows that "organ printing" with cellular spheroids can establish a precise 3D geometry and mimic basic organ architectures (Jakab *et al*., 2004a; Jakab *et al*., 2004b).

In fact, a well-defined geometry allows the modelling of dynamic processes, such as organ formation and growth (Glicklis, Merchuk & Cohen, 2004), diffusion of drugs, cell invasion, as well as angiogenesis (Jiang *et al*., 2005; Stein *et al*., 2007; Tabatabai, Williams & Bursac, 2005). Spheroids are already well-established in clinical research, especially as models of small solid tumors (Sutherland, 1988; Sutherland, McCredie & Inch, 1971). They are the system of choice for therapeutically-oriented biomedical studies (Mueller-Klieser, 1997; Sutherland, 1988; Sutherland *et al*., 1971), and have been applied in biotechnology, e.g. in tissue engineering of human bone (Kale *et al*., 2000). With current advances in automation, microscopy imaging, and processing of large amount of data, cellular spheroids will become increasingly important for *in vitro* drug discovery screening and toxicity assays.

ORGANOTYPIC CULTURES

Organotypic cultures allow testing the functions of an organ exposed to toxicants by employing the actual organ itself grown *in vitro* under tissue culture conditions (Holopainen, 2005). This allows the maintenance of the tissue's architecture. Organotypic conditions are obtained by culturing organ slices of microscopic thickness (*Figure 1d*). Slices are cultured on semiporous membranes at the air-liquid interface, on collagen-coated substrates, or within 3D collagen. Each cell in the slice is less than a few hundred micrometers away from the media and oxygen supply. Thus, the tissue slice remains viable for many weeks or months even in serum-free media.

Organotypic lung, skin, and brain cultures have been developed and are widespread in drug discovery and toxicology. Organotypic brain slice cultures are well established for neurotoxicological screening and toxicological mechanistic research (Noraberg, 2004). Ootani *et al*. developed a promising combination of 3D culture in collagen hydrogel and organotypic culture. They realized a 3D intestinal model with murine intestinal stem cells (ISC), employing explants as starting material. Intestine explants were cultured within 3D collagen I hydrogel maintained at the air-liquid interface. On day seven, cystic structures (termed "intestinal spheres") appeared. Ootani *et al*. observed that the intestinal sphere is a polarized epithelial monolayer expressing the markers of intestine epithelium. They have shown that ISC in 3D culture differentiated to absorptive enterocytes and Goblet cells. The myofibroblasts present in the explants (the candidate ISC niche) supported long-term (i.e. for over 350 days) proliferation and differentiation of the tissue. This intestinal organotypic culture could have a significant impact on drug and toxicity assays.

In summary, the major advantages of whole organ slices are that the basic organ architecture is retained, and that the inter-individual variability is maintained. The drawbacks are the need of numerous biopsies and the difficult standardization of the assays.

3D CULTURES ON POROUS SUBSTRATES AND MICROCARRIERS

CULTURES ON POROUS MEMBRANES

Epithelial structures such as human skin models have been developed as 3D cultures on porous membrane ("filter-well inserts", e.g. Millicell from Millipore, Billerica, MA, USA or Transwell from Nunc, Rochester, NY, USA) or on polymeric fibre mesh (*Figure 1c-1*). The epithelial cells are cultured to confluence at the air-liquid interface. In this configuration, cells differentiate to polarized epithelial sheets (Justice, Badr & Felder, 2009). Sun *et al*. developed a 3D skin model by culturing the human keratinocyte cell line HaCaT on a biologically inert non-hydrolysable commercial scaffold (non-woven viscose rayon, trade name Azowipes™) (Sun *et al*., 2006). The ability of the skin cells to respond to toxic xenobiotics (hydrogen peroxide and silver nitrate) in 3D and 2D was compared. The results showed that the concentration of H_2O_2 and $AgNO_3$ necessary to produce a 50% loss of viability (IC_{50}) was doubled in 3D compared to 2D. Consistent results were obtained by comparing the IC_{50} of human dermal fibroblasts and endothelial cells 3D and 2D cultures. The improved viability of 3D cultures vs. 2D ("multicellular resistance") has been observed by other researchers that employed 3D macroporous hydrogel as a substrate (Dainiak *et al*., 2008), and is consistent with the higher resistance to cytotoxic drugs observed *in vivo* (Desoize & Jardillier, 2000). These data suggest that the outcome of toxicological assays obtained with 2D cultures needs to be critically reassessed (Sun *et al*., 2006). Human colon carcinoma cells (Caco2) cultured for 14-20 days on a porous membrane establish intestinal cell polarity, tight junction, and transport properties. These differentiated Caco2 cells are morphologically and functionally similar to intestinal enterocytes. This is a valuable in vitro system to screen the intestinal absorption of drugs and toxicants (Bohets *et al*., 2001). A drawback is that Caco2 cells represent enterocytes only. A more realistic intestinal model includes the adenocarcinoma cells

HT29. These cells differentiate into mature Goblet cells under the influence of the drug methotrexate (Behrens *et al*., 2001). The mucus-secreting Goblet cells represent the second major cell type in the intestine.

Some 3D cell cultures on porous membrane mimicking epidermis and other epithelial organs have been fully validated for toxicity testing and will be extensively discussed in the paragraph "3D cultures in toxicology".

CULTURES ON MICROCARRIERS

Differentiated epithelia can also be obtained with microcarriers (*Figure 1c-2,3*). Microcarriers are polymeric beads with a diameter of 300-500 μm. Microcarriers provide an enourmous surface to volume ratio and support cell differentiation similarly to porous membranes. A commercially available microcarrier is the gelatin-coated Cytodex 3 (GE Healthcare, Chalfont St. Giles, UK). The cell-seeded microcarriers can be cultured in a rocked dish or in a rotating well vessel (see following paragraph). An advantage is that cells on microcarriers are maintained in homogeneous liquid medium during culture. This minimizes sample-to-sample variability. A further advantage is that the microcarrier system can be easily scaled-up to a high-throughput format (Justice *et al*., 2009).

3D CELL CULTURES IN TOXICOLOGY

The European REACH-agenda will re-evaluate tens of thousands of chemical substances. This will be impossible without the use of *in vitro* assays. Moreover, EU regulation has already banned the use of animals for toxicological testing in the cosmetic industry since 2009 (76/768/EEC, February 2003). Assays based on 3D culture will have a pivotal role in replacing animals (Bhogal *et al*., 2005). However, the validation of new toxicity assays for regulatory purposes is a technically demanding process, very expensive and very time-consuming. Therefore, it is no surprise that just few 3D cell models have been validated so far by authorities, such as the European Center for the Validation of Alternative Methods (ECVAM, http://ecvam.jrc.it/).

ASSAYS WITH 3D EPIDERMAL MODELS

Three commercial epidermal models have been fully validated, namely EPISKIN™ (L'Oréal, www.invitroskin.com), EpiDerm™ (MatTek, www.mattek.com) and SkinEthic™ (www.skinethic.com) (Bhogal *et al*., 2005; Netzlaff *et al*., 2005). In all the three models, several stratified layers of epidermal cells are cultured at the air-liquid interface on microporous filter inserts (such as MillicellCM by Millipore or Nunc polycarbonate inserts). Primary normal human keratinocytes are employed. The cells are obtained from donor specimens and expanded in monolayer culture to provide large pools. The general morphology, lipid composition, and biochemical markers of the 3D epidermis models are close to that of human skin. A common problem of these models is the higher permeability compared to human skin (Netzlaff *et al*., 2005). Protocols to discriminate between corrosive and non-corrosive substances on skin based on EPISKIN and EpiDerm have been validated by ECVAM, and are

now accepted as EU test guidelines (Bhogal *et al*., 2005; Kandarova *et al*., 2004; Kandarova *et al*., 2005). 3D models of human corneal epithelium, tracheal/bronchial epithelium, buccal and gingival mucosa, and vaginal cervical mucosa are also commercially available (MakTek, SkinEthic). All the systems are based on primary normal human cells.

ASSAYS WITH LIVER MODELS

While 3D epidermis models provide essential data on skin corrosivity and phototoxicity, they are not suitable for metabolite toxicity or organ-specific toxicity, such as liver toxicity. Liver toxicity is the first cause of failing of a drug candidate during the clinical phase, and of withdrawal of a drug from the market (Kaplowitz, 2005). Liver models suitable for toxicity testing should maintain the liver functionality for up to several weeks. Particularly, the expression of cytochrome P450-mixed function monooxygenases (CYPs, the most important group of chemicals-metabolizing enzymes in the liver) must be conserved. Liver models based on 3D cell cultures have been intensively investigated. A broad range of 3D cell culture techniques has been employed to develop liver models, including culture in hydrogels, porous membrane, and cellular spheroids.

LIVER MODELS WITH 3D HYDROGELS

Lee *et al*. developed a microarray ("DataChip") for high-throughput toxicity assay based on Hep3B cells (a human hepatocellular carcinoma cell line that partially retains liver-specific functions) embedded in 3D hydrogels (Lee *et al*., 2008). The cells were encapsulated in microdroplets of alginate gel. Each microdroplets contained ~60 cells. The droplets were spotted onto glass slides producing a 1080-spots array. Alginate is not degraded by matrix metalloproteinases produced by cells. Thus, the droplets were stable over several days, and their volume was as small as 20 nanoliters. This allowed increasing the density of the spots in the array. The toxicity of CYPs-metabolites was tested with the 3D cell microarray, as a proof of principle for toxicity screening of drugs and drugs metabolites. Dose-response curves were measured on 27 CYPs-metabolized substances, including digoxin, doxorubicin, acetaminophen, and ketokonazole. The assay was able to predict the influence of CYPs metabolism on the toxicity of this diverse range of xenobiotics.

LIVER MODELS WITH POROUS MEMBRANES

A sandwich culture of rat primary hepatocytes employing synthetic ECM was developed by Du *et al*. (Du *et al*., 2008). The culture was maintained in a commercial small-sized perfusion bioreactor (Minusheet carriers, Minucell GmbH, Germany), suitable for metabolism/toxicity assays. The synthetic ECM consisted of two porous membranes sandwiching the hepatocytes. A galactosilated polyethylene-terephtalate (PET-Gal) was employed as bottom (basal) substrate for the hepatocytes. A polyethylene porous membrane coated with the oligopeptide Gly-Arg-Gly-Asp-Ser (PET-GRGDS) was employed as top (apical) support. The two synthetic basal and apical

membranes mimic the space of Disse in liver. The authors compared the performance of the PET-Gal/PET-GRGDS system and the collagen sandwich system. The parameters examined in the comparison were the establishment of *in vivo*-like cell polarity, cell-cell interaction, biliary excretion, differentiated liver function, and mass transfer of medium and metabolites. The authors observed that PET-Gal/PET-GRGDS sandwich has a similar or improved performance compared to the collagen sandwich system. Hepatocyte polarity was established in both systems, as confirmed by the cortical rearrangement of the F-actin cytoskeleton and formation of bile canaliculi. Also the biliary excretion measured with fluorescein diacetate was comparable. Interestingly, liver functionality parameters, i.e. albumin and urea production, as well as cytochrome P540 1A activity were significantly higher in the PET-Gal/PET-GRGDS sandwich than in the collagen sandwich for over 14 days. The improvement of in vivo-like liver functionality in the PET-Gal/PET-GRGDS system could be due to better cell-cell contacts and more efficient diffusion of nutrients and waste removal.

SPHEROID-BASED LIVER MODELS

Hepatocyte spheroids have been produced by (Brown *et al*., 2003) and maintained in a differentiated state for several weeks by culturing in a rotating well vessel (RWV, www.synthecon.com). The spheroids were obtained by culturing primary rat hepatocytes in 100-mm Petri dishes constantly shacked with an orbital shaker. Then, the spheroids were further cultured in a RWV. The hepatocyte spheroids remained viable for several weeks in the RWV and maintained specific liver functions. Phase 1 and phase 2 CYPs xenobiotics metabolism was confirmed by measuring the activity of CYP 2B1/2, CYP 2E1, CYP 2D and CYP 3A. Prolonged albumin secretion was also observed. Thus, RWV is well suited for long-term culture of liver cell spheroids, with good viability and maintenance of liver-specific functions. With this system, toxicological and pharmacological assays are possible.

Xu *et al*. compared primary primary rat hepatocytes spheroids and HepG2 (a human hepatocellular carcinoma cell line) spheroids as in vitro models for toxicity assays (Xu, Ma & Purcell, 2003c). The authors claim that HepG2 spheroids are suitable for *in vitro* toxicity studies, and produce results comparable with that obtained with primary hepatocytes.

FURTHER SPHEROID-BASED ASSAYS

Kloss *et al*. developed a spheroids biochip for toxicological screening based on impedance spectroscopy (Kloss *et al*., 2008). The frequency-dependent impedance spectrum provides information on the dielectric and structural properties of the spheroids. Single spheroids were trapped into pyramidal cavities with a size of 300 μm etched on a silicon wafer. Each cavity contained four gold microelectrodes to measure the spheroid's impedance. Each biochip contained 25 cavities. The biochip was used to assess the effect of drugs on cell packaging density, surface structure, and ECM architecture of the spheroids. Spheroids obtained from human melanoma cells (Bro), african green monkey kidney (Cos-7), chinese hamster ovary cells (CHO), and chicken retina cells were tested. The most compact spheroids (Bro) showed low impedance,

while less compact and irregular chicken retina cell spheroids displayed the highest impedance. Human melanoma (Bro) spheroids were employed for a screening assay with cytotoxic drugs. After eight hours of exposition, the drugs forskolin, staurosporine and campthotecin caused an increase of the impedance, while doxorubicin and tamoxifen induced a decrease of the impedance. Correlating the change of the impedance with specific drug-induced effect requires additional investigation, e.g. with light microscopy. Previous work on spheroid impedance spectra suggests that changes in the spheroid's compactness (Kloss *et al*., 2008) as well as disintegration of the cell's membrane (Thielecke, Mack & Robitzki, 2001) are the parameters with the largest influence on the impedance.

Further works have shown that impedance assays on embryonic chicken heart muscle spheroids are suitable for drug and toxicity testing (Reininger-Mack, Thielecke & Robitzki, 2002). Thus, spheroid-based biochips with impedance/potential recording are promising for toxicity assay for food quality control, environmental control, pharmaceutical and cosmetic industry.

In summary, several toxicity assays based on 3D cell models have been developed and are promising in their ability to mimic specific organ response to toxicants. However, the validation of these assays from the research stage to real-world application is a long and cumbersome process. This is evidenced by the fact that just three skin models have been fully validated so far to replace animal testing. A close cooperation between regulatory authorities, industry, and academia is required in order to speed up the validation process.

Conclusions and prospects

NOVEL EXTRACELLULAR MATRICES

The successful introduction of toxicity screenings based on 3D cell cultures strongly depends on the availability of 3D matrices that are easy to handle and with low price. Reproducible and standardized matrices designed to mimic specific tissue microenvironments are required. A broad range of synthetic scaffolds developed for tissue engineering is available (Kim & Mooney, 1998). Substantial efforts have been devoted to increase tissue-like specificity and biocompatibility of 3D hydrogels. In the near future, novel 3D matrices will contribute to the wider adoption of 3D cell cultures for large-scale toxicity screenings.

EXTRACTED ECM HYDROGELS

Collagen I and basement membrane extract alone do not accurately mimic the composition of the different ECM types found in tissues (Uriel *et al*., 2008). Laminin-1 represents a minor fraction of the ECM proteins found in most adult tissues. Further laminin isoforms or basement membrane components are essential to recapitulate specific property of tissues *in vitro* (Uriel *et al*., 2008). A procedure to extract ECM proteins from selected tissues has been elaborated by (Uriel *et al*., 2008) and (Brey *et al*., 2007). The source tissue is decellularized by treating with EDTA, with dispase, or by grinding. Then, the basement membrane is homogenized in a high-salt solu-

tion containing a protease inhibitor. Finally, the basement membrane proteins are extracted by soaking in presence of urea. The extracted ECM proteins can be gelled by rising pH or temperature. The resulting gel is a highly specific duplicate of the tissue's ECM.

SYNTHETIC ECM HYDROGELS

Extracted hydrogels recapitulates many *in vivo* characteristic of the ECM. However, they may contain non quantified impurities, such as growth factors and intracellular proteins. Major issues are batch-to-batch variations and limited availability. Fully synthetic hydrogels for 3D cell-culture would reduce costs and improve the reproducibility of results. Zhang *et al*. introduced nanofibers hydrogels based on self-assembling oligopeptides with repeating motifs Glu-Ala-Lys or Arg-Ala-Asp (Zhang *et al*., 2005). The oligopeptide is available on the market with the trade name "Puramatrix" (BD Biosciences) (Zhang *et al*., 1997). Nanofiber self-assembly is triggered by mixing the peptide solution with culture medium at physiological pH (~7). The nanofibers have a diameter of about 10 nm and the gel pore size is between 5 and 200 nm (Zhang *et al*., 2005). The nanofiber gel surrounds the cells similarly to the natural ECM. Ulijn *et al*. introduced short oligopeptides with the motifs Phe-Phe, Fmoc-Leu-Leu-Leu, as well as Fmoc-X-Phe-Phe (X=Ala, Val, Leu, Phe) (Ulijn *et al*., 2007). These oligopeptides form stable hydrogels that support cell growth in 3D. The drawback of fully synthetic hydrogels is that they lack cell-specific adhesion-promoting sites. Thus, ECM proteins such as laminin-1 or fibronectin must be added in order to promote cell growth and differentiation. Semi-synthetic hydrogels consisting of cross-linked derivatives of hyaluronan and gelatine are a promising alternative to both animal-derived and fully synthetic hydrogels (Serban, Scott & Prestwich, 2008). Hyaluronan is a major constituent of natural ECM, while gelatine is denatured collagen. In the semisynthetic hydrogel, both hyaluronan and gelatine are modified with reactive thiol groups. Polyethylene glycol diacrylate is employed as cross-linker. Studies have shown that primary hepatocytes cultured in the 3D hyaluronan-gelatine matrix maintain liver-specific functionality longer than 2D-cultured hepatocytes (Prestwich *et al*., 2007). A hyaluronan semi-synthetic matrix is available commercially (trade name "Extracel", Glycosan Biosystems).

Alginate hydrogels are also widely employed in tissue engineering (Lee & Mooney, 2001). Gelatine-coated alginate microcarriers are emerging as a promising matrix for large-scale 3D cell cultures (Justice *et al*., 2009).

Recently, macroporous hydrogels (cryogels) have been applied to 3D cell cultures (Plieva *et al*., 2008a). Cryogels are prepared by gelation at sub-zero temperature. They have a well-controlled porosity, determined by the size of the growing ice crystals. The cryogel surface can be easily functionalized with ECM molecules, such as collagen I or RGD peptides (Dainiak *et al*., 2008). The porous structure of 3D cryogels, as well as tissue-like elasticity, allows for a physiological cell microenvironment (Plieva *et al*., 2008b). 3D cryogel scaffolds have been adapted to standard 96-well format. A drug toxicity test performed with this system has shown that human colon cancer cells (HCT116) are 3.5-fold less sensitive to cisplatin than the same cells cultured in 2D (Dainiak *et al*., 2008).

Photopolymerizable polyethylene glycol diacrylate (PEG-DA) hydrogels supplemented with ECM molecules have been employed to fabricate 3D liver cell constructs. PEG-based hydrogels are biocompatible, hydrophilic, and can easily be functionalized. By using micro-photolithography, Liu Tsang *et al*. patterned a three-layer hexagonal multilayer structure with PEG-DA embedding hepatocytes (Liu Tsang *et al*., 2007). The hexagonal geometry mimics the branching architecture of liver *in vivo* and optimizes the exchange of nutrients and waste in the perfusion system. The study showed that hepatocytes can be maintained for two weeks in the perfused PEG-DA. The fabrication of photopatterned multilayer 3D cultures could be straightforwardly extended to other cell types, realizing miniaturized "printed organs" suitable for toxicology screening.

LIGHT-SHEET BASED FLUORESCENCE MICROSCOPY

The possibility to perform high-content screening (HCS) of 3D cell cultures is essential to accomplish efficient and predictive toxicity assays and replace testing on animals. HCS harnesses fluorescence images of cells with algorithms that track the spatiotemporal distribution of target proteins. HCS can probe events in live cells (e.g. sub-cellular protein translocation) that are not accessible in conventional high throughput screening, and is establishing itself as a core technology in cell-based toxicity assays (Rausch, 2006). However, 3D cell cultures are challenging for optical microscopy. Wide-field epifluorescence microscopy and confocal microscopy work optimally on flat specimens such as 2D cultured cells, but meet their limits when applied to 3D cell cultures. This is due to large size (from hundreds of micrometers to few millimeters), strong light scattering due to the ECM fibrous network, and high cell density. Further serious issues are phototoxicity as well as photobleaching due to high-intensity illumination of the specimen. Other imaging techniques, such as the environmental scanning electron microscope (ESEM), have been successfully applied to 3D cultures (Uroukov & Patton, 2008), but they are not suitable for large-scale screening due to slow specimen preparation. Recent advances in imaging technology are removing some of the obstacles that have prevented the observation of live cells that grow in a more natural, physiological three-dimensional environment. The Light Sheet-based Fluorescence Microscope (LSFM) (*Figure 2A*) is optimally suited for imaging of 3D cell cultures. Two implementations of LSFM exist: Single Plane Illumination Microscope (SPIM) (Greger, Swoger & Stelzer, 2007; Huisken *et al*., 2004; Pampaloni *et al*., 2007), and Digital Scanned Light-sheet Microscope (DSLM) (Keller *et al*., 2008). In LSFM, the specimen is selectively illuminated in the focal plane with a laser light-sheet. Thus, fluorescence emission is excited exclusively in a thin plane in the specimen. The out-of-plane regions are not illuminated. The fluorescence from the illuminated plane is collected with an objective lens placed perpendicularly to the light-sheet and imaged with a sensitive CCD camera. The objective lens focal plane and the illuminated plane overlap, so that only the plane that is illuminated is imaged. A 3D image stack is obtained by moving the specimen through the light-sheet. Adjacent planes can be as close as 0.5 micrometers. Additionally, the specimen can be axially rotated. This allows recording 3D image stacks of the same specimen from different angles. By using high-numerical aperture

water-dipping objective lenses (e.g. with NA=0.85 or NA=1.0), a resolution at sub-cellular level can be achieved. LSFM has a large penetration depth also in strongly scattering specimens, very low photobleaching, and high recording speed. Typical specimens for LSFM are 3D cell cultures in ECM gel, cellular spheroids (*Figure 2B-C, Figure 3*), zebrafish and medaka fish embryos, drosophila embryos, as well as tissue explants. The specimen handling and preparation for 3D imaging is straightforward for most types of specimens. Cellular spheroids are just embedded in 0.5% agarose before imaging (*Figure 2B*). Simple mounting procedures, fast recording speed, and high-resolution multi-channel fluorescence imaging allow the fast screening of a large number of spheroids, tissue explants, and cell cultures. We anticipate that LSFM will greatly advance toxicity assays, allowing HCS and HCA of size, shape, texture, and intensity at individual cell level in 3D cell cultures.

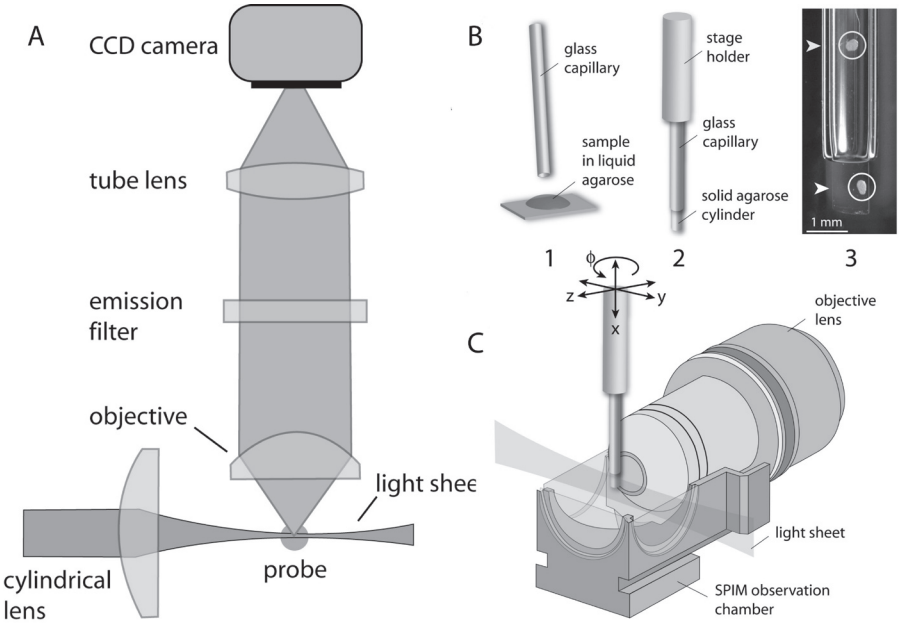

Figure 2. LSFM set-up. (A) In an LSFM, a laser light sheet is fed into the specimen from the side and observed at an angle of 90° to the illumination optical axis. In this implementation (Single Plane Illumination Microscope, SPIM (Greger *et al*., 2007; Huisken *et al*., 2004)), the light sheet is produced by a cylindrical lens. The optical sectioning arises from the overlap of the focal plane of the fluorescence detection system with the central plane of the excitation light sheet. Light-sheet-based fluorescence microscopes perform particularly well with long working distance lenses. Since the numerical aperture of the illumination system is much smaller than that of the detection system, light-sheet-based fluorescence microscopes have a good penetration depth. Millimetre-sized specimens can be observed in their totality. A further increase in resolution and information content can be obtained by observing the same specimen multiple times but along different directions. Parts of the sample that would otherwise be hidden or obscured along one direction now become visible. In a further step, the images stacks that are independently recorded along different angles are combined to yield a single fused images stack. (B1) The spheroid is inside a droplet of liquid agarose and sucked into a glass capillary. (B2) As soon as the agarose has formed a gel, the sample is pushed out of the capillary and imaged. (B3) A photograph of the specimen with the spheroid marked by the circle at the bottom. A second spheroid within the capillary is found further up (upper circle). The white arrow points at the boundary of the agarose. The yellow arrow indicates the boundary of the capillary.

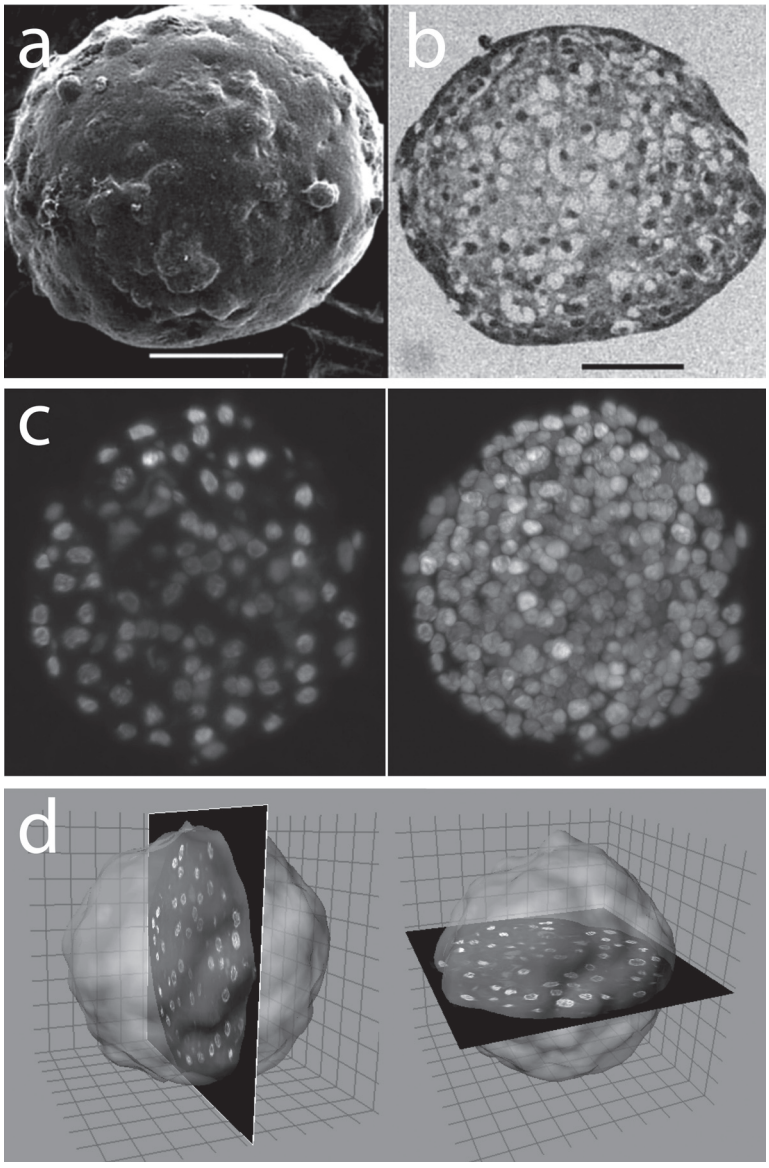

Figure 3. (A) An image of a liver spheroid recorded by scanning electron microscope. The bar represents 50 μm. (B) The histological section of a six-days old spheroid. Scale bar 50 μm. (C) Fluorescence image of a cellular pancreas tumour cells (BxCP-3) spheroid. The image was recorded with Light-sheet based fluorescence microscopy (SPIM). The image is calculated by combining twelve views recorded along different angles. The cell nuclei are labelled with the fluorescent dye Draq5™. The spheroid diameter is ~100 μm. (C, left) A three-dimensional maximum projection of about 100 single slices spaced 0.5 μm apart. (C, right) A single slice showing the central region of the spheroid. (D) The same spheroid rendered in three dimensions with the image processing software Imaris™.

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References

Behrens, I., Stenberg, P., Artursson, P. & Kissel, T. (2001). Transport of lipophilic drug molecules in a new mucus-secreting cell culture model based on HT29-MTX cells. *Pharm. Res.* 18, 1138-45.

Beningo, K. A., Dembo, M. & Wang, Y. L. (2004). Responses of fibroblasts to anchorage of dorsal extracellular matrix receptors. *Proc. Natl. Acad. Sci. USA* **101,** 18024-9.

Bessea, L., Coulomb, B., Lebreton-Decoster, C. & Giraud-Guille, M. M. (2002). Production of ordered collagen matrices for three-dimensional cell culture. *Biomaterials* **23,** 27-36.

Bhadriraju, K. & Chen, C. S. (2002). Engineering cellular microenvironments to improve cell-based drug testing. *Drug Discov. Today* **7,** 612-20.

Bhogal, N., Grindon, C., Combes, R. & Balls, M. (2005). Toxicity testing: creating a revolution based on new technologies. *Trends Biotechnol.* **23,** 299-307.

Bilodeau, K. & Mantovani, D. (2006). Bioreactors for tissue engineering: focus on mechanical constraints. A comparative review. *Tissue Eng.* **12,** 2367-2383.

Birgersdotter, A., Sandberg, R. & Ernberg, I. (2005). Gene expression perturbation in vitro--a growing case for three-dimensional (3D) culture systems. *Semin. Cancer Biol.* **15,** 405-12.

Bissell, M. J. & Radisky, D. (2001). Putting tumours in context. *Nat. Rev. Cancer* **1,** 46-54.

Bohets, H., Annaert, P., Mannens, G., Van Beijsterveldt, L., Anciaux, K., Verboven, P., Meuldermans, W. & Lavrijsen, K. (2001). Strategies for absorption screening in drug discovery and development. *Curr. Top. Med. Chem.* **1,** 367-83.

Brey, E. M., Uriel, S., Labay, E. & Weichselbaum, R. R. (2007). Tissue-specific basement membrane gels. US Patent App. 11/585,353, 2006.

Brown, L. A., Arterburn, L. M., Miller, A. P., Cowger, N. L., Hartley, S. M., Andrews, A., Silber, P. M. & Li, A. P. (2003). Maintenance of liver functions in rat hepatocytes cultured as spheroids in a rotating wall vessel. *In vitro Cell Dev. Biol. Anim*. **39,** 13-20.

Chevallay, B. & Herbage, D. (2000). Collagen-based biomaterials as 3D scaffold for cell cultures: applications for tissue engineering and gene therapy. *Med. Biol. Eng. Comput.* **38,** 211-8.

Cukierman, E., Pankov, R., Stevens, D. R. & Yamada, K. M. (2001). Taking cell-matrix adhesions to the third dimension. *Science* **294,** 1708-1712.

Dainiak, M. B., Savina, I. N., Musolino, I., Kumar, A., Mattiasson, B. & Galaev, I. Y. (2008). Biomimetic macroporous hydrogel scaffolds in a high-throughput screening format for cell-based assays. *Biotechnol. Prog.* **24,** 1373-83.

de Boo, J. & Hendriksen, C. (2005). Reduction strategies in animal research: a review of scientific approaches at the intra-experimental, supra-experimental and extra-experimental levels. *Altern. Lab. Anim.* **33,** 369-77.

Desoize, B. & Jardillier, J. (2000). Multicellular resistance: a paradigm for clinical resistance? *Crit Rev. Oncol. Hematol*.**36,** 193-207.

Du, Y., Han, R., Wen, F., Ng San San, S., Xia, L., Wohland, T., Leo, H. L. & Yu, H. (2008). Synthetic sandwich culture of 3D hepatocyte monolayer. *Biomaterials* **29,** 290-301.

Foth, H. & Hayes, A. (2008). Concept of REACH and impact on evaluation of chemicals. *Hum. Exp. Toxicol.* **27,** 5-21.

Friedrich, J., Seidel, C., Ebner, R. & Kunz-Schughart, L. A. (2009). Spheroid-based drug screen: considerations and practical approach. *Nat. Protoc.* **4,** 309-24.

Ghosh, S., Spagnoli, G. C., Martin, I., Ploegert, S., Demougin, P., Heberer, M. & Reschner, A. (2005). Three-dimensional culture of melanoma cells profoundly affects gene expression profile: a high density oligonucleotide array study. *J. Cell Physiol*. **204,** 522-31.

Glicklis, R., Merchuk, J. C. & Cohen, S. (2004). Modeling mass transfer in hepatocyte spheroids via cell viability, spheroid size, and hepatocellular functions. *Biotechnol. Bioeng.* **86,** 672-80.

Glicklis, R., Shapiro, L., Agbaria, R., Merchuk, J. C. & Cohen, S. (2000). Hepatocyte behavior within three-dimensional porous alginate scaffolds. *Biotechnol. Bioeng*. **67,** 344-53.

Gomez-Lechon, M. J., Jover, R., Donato, T., Ponsoda, X., Rodriguez, C., Stenzel, K. G., Klocke, R., Paul, D., Guillen, I., Bort, R. & Castell, J. V. (1998). Long-term expression of differentiated functions in hepatocytes cultured in three-dimensional collagen matrix. *J. Cell Physiol.* **177,** 553-562.

Greger, K., Swoger, J. & Stelzer, E. H. (2007). Basic building units and properties of a fluorescence single plane illumination microscope. *Rev. Sci. Instrum.* **78,** 023705.

Hasspieler, B., Haffner, D., Stelljes, M. & Adeli, K. (2006). Toxicological assessment of industrial solvents using human cell bioassays: assessment of short-term cytotoxicity and long-term genotoxicity potential. *Toxicol. Ind. Health* **22,** 301-15.

Holopainen, I. E. (2005). Organotypic hippocampal slice cultures: a model system to study basic cellular and molecular mechanisms of neuronal cell death, neuroprotection, and synaptic plasticity. *Neurochem. Res.* **30,** 1521-8.

Huisken, J., Swoger, J., Del Bene, F., Wittbrodt, J. & Stelzer, E. H. (2004). Optical sectioning deep inside live embryos by selective plane illumination microscopy. *Science* **305,** 1007-9.

Ivascu, A. & Kubbies, M. (2006). Rapid generation of single-tumor spheroids for high-throughput cell function and toxicity analysis. *J. Biomol. Screen.* **11,** 922-932.

Jakab, K., Neagu, A., Mironov, V. & Forgacs, G. (2004a). Organ printing: fiction or science. *Biorheology* **41,** 371-5.

Jakab, K., Neagu, A., Mironov, V., Markwald, R. R. & Forgacs, G. (2004b). Engineering biological structures of prescribed shape using self-assembling multicellular systems. *Proc. Natl. Acad. Sci. USA* **101,** 2864-9.

Jessberger, S., Clemenson, G. D., Jr. & Gage, F. H. (2007). Spontaneous fusion and nonclonal growth of adult neural stem cells. *Stem Cells* **25,** 871-4.

Jiang, Y., Pjesivac-Grbovic, J., Cantrell, C. & Freyer, J. P. (2005). A multiscale model for avascular tumor growth. *Biophysical Journal* **89,** 3884-3894.

Justice, B. A., Badr, N. A. & Felder, R. A. (2009). 3D cell culture opens new dimensions in cell-based assays. *Drug Discov. Today* **14,** 102-7.

Kale, S., Biermann, S., Edwards, C., Tarnowski, C., Morris, M. & Long, M. W. (2000). Three-dimensional cellular development is essential for ex vivo formation of human bone. *Nat.Biotechnol.* **18,** 954-8.

Kandarova, H., Liebsch, M., Genschow, E., Gerner, I., Traue, D., Slawik, B. & Spielmann, H. (2004). Optimisation of the EpiDerm test protocol for the upcoming ECVAM validation study on in vitro skin irritation tests. *Altex* **21,** 107-14.

Kandarova, H., Liebsch, M., Gerner, I., Schmidt, E., Genschow, E., Traue, D. & Spielmann, H. (2005). The EpiDerm test protocol for the upcoming ECVAM validation study on in vitro skin irritation tests--an assessment of the performance of the optimised test. *Altern. Lab. Anim.* **33,** 351-67.

Kaplowitz, N. (2005). Idiosyncratic drug hepatotoxicity. *Nat. Rev. Drug Discov.* **4,** 489-99.

Keller, P. J., Schmidt, A. D., Wittbrodt, J. & Stelzer, E. H. (2008). Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. *Science* **322,** 1065-9.

Kelm, J. M., Timmins, N. E., Brown, C. J., Fussenegger, M. & Nielsen, L. K. (2003). Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnol. Bioeng.* **83,** 173-180.

Kim, B. S. & Mooney, D. J. (1998). Development of biocompatible synthetic extracellular matrices for tissue engineering. *Trends Biotechnol.* **16,** 224-30.

Kleinman, H. K. & Martin, G. R. (1989). Reconstituted basement membrane complex with biological activity. US Patent 4,829,000, 1989

Kloss, D., Fischer, M., Rothermel, A., Simon, J. C. & Robitzki, A. A. (2008). Drug testing on 3D in vitro tissues trapped on a microcavity chip. *Lab Chip* **8,** 879-84.

Knight, A. (2007a). Animal experiments scrutinised: systematic reviews demonstrate poor human clinical and toxicological utility. *Altex* **24,** 320-5.

Knight, A. (2007b). Systematic reviews of animal experiments demonstrate poor human clinical and toxicological utility. *Altern. Lab. Anim.* **35,** 641-59.

Korff, T. & Augustin, H. G. (1998). Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. *J. Cell Biol.* **143,** 1341-52.

Kunz-Schughart, L. A., Freyer, J. P., Hofstaedter, F. & Ebner, R. (2004). The use of 3-D cultures for high-throughput screening: the multicellular spheroid model. *J. Biomol. Screen* **9,** 273-85.

Layer, P. G., Robitzki, A., Rothermel, A. & Willbold, E. (2002). Of layers and spheres: the reaggregate approach in tissue engineering. *Trends Neurosci.* **25,** 131-4.

Lazar, A., Mann, H. J., Remmel, R. P., Shatford, R. A., Cerra, F. B. & Hu, W. S. (1995). Extended liver-specific functions of porcine hepatocyte spheroids entrapped in collagen gel. *In Vitro Cell Dev. Biol. Anim.* **31,** 340-6.

Lee, K. Y. & Mooney, D. J. (2001). Hydrogels for tissue engineering. *Chem. Rev* .**101,** 1869-79.

Lee, M. Y., Kumar, R. A., Sukumaran, S. M., Hogg, M. G., Clark, D. S. & Dordick, J. S. (2008). Three-dimensional cellular microarray for high-throughput toxicology assays. *Proc. Natl. Acad. Sci. USA* **105,** 59-63.

Li, A. P. (2001). Screening for human ADME/Tox drug properties in drug discovery. *Drug Discov. Today* **6,** 357-366.

Li, S., Lao, J., Chen, B. P., Li, Y. S., Zhao, Y., Chu, J., Chen, K. D., Tsou, T. C., Peck,

K. & Chien, S. (2003). Genomic analysis of smooth muscle cells in 3-dimensional collagen matrix. *Faseb. J.* **17,** 97-9.

Lilienblum, W., Dekant, W., Foth, H., Gebel, T., Hengstler, J. G., Kahl, R., Kramer, P. J., Schweinfurth, H. & Wollin, K. M. (2008). Alternative methods to safety studies in experimental animals: role in the risk assessment of chemicals under the new European Chemicals Legislation (REACH). *Arch. Toxicol.* **82,** 211-36.

Lin, J., Sahakian, D. C., de Morais, S. M., Xu, J. J., Polzer, R. J. & Winter, S. M. (2003). The role of absorption, distribution, metabolism, excretion and toxicity in drug discovery. *Curr. Top. Med. Chem.* **3,** 1125-54.

Liu Tsang, V., Chen, A. A., Cho, L. M., Jadin, K. D., Sah, R. L., DeLong, S., West, J. L. & Bhatia, S. N. (2007). Fabrication of 3D hepatic tissues by additive photopatterning of cellular hydrogels. *Faseb J .***21,** 790-801.

Lutolf, M. P. & Hubbell, J. A. (2005). Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat. Biotechnol.* **23,** 47-55.

Mazzoleni, G., Di Lorenzo, D. & Steimberg, N. (2009). Modelling tissues in 3D: the next future of pharmaco-toxicology and food research? *Genes Nutr.* **4,** 13-22.

Montesano, R. (1986). Cell-extracellular matrix interactions in morphogenesis: an in vitro approach. *Experientia* **42,** 977-85.

Moscona, A. (1961). Rotation-mediated histogenetic aggregation of dissociated cells. A quantifiable approach to cell interactions in vitro. *Exp. Cell Res.* **22,** 455-475.

Mostov, K., Su, T. & ter Beest, M. (2003). Polarized epithelial membrane traffic: conservation and plasticity. *Nat. Cell Biol.* **5,** 287-293.

Mueller-Klieser, W. (1997). Three-dimensional cell cultures: from molecular mechanisms to clinical applications. *Am. J. Physiol.* **273,** C1109-23.

Netzlaff, F., Lehr, C. M., Wertz, P. W. & Schaefer, U. F. (2005). The human epidermis models EpiSkin, SkinEthic and EpiDerm: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. *Eur. J. Pharm. Biopharm.* **60,** 167-78.

Noraberg, J. (2004). Organotypic brain slice cultures: an efficient and reliable method for neurotoxicological screening and mechanistic studies. *Altern. Lab. Anim.* **32,** 329-37.

O'Brien, L. E., Zegers, M. M. & Mostov, K. E. (2002). Opinion: Building epithelial architecture: insights from three-dimensional culture models. *Nat. Rev. Mol. Cell Biol.* **3,** 531-537.

Olson, H., Betton, G., Robinson, D., Thomas, K., Monro, A., Kolaja, G., Lilly, P., Sanders, J., Sipes, G., Bracken, W., Dorato, M., Van Deun, K., Smith, P., Berger, B. & Heller, A. (2000). Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regul. Toxicol. Pharmacol.* **32,** 56-67.

Pampaloni, F., Reynaud, E. G. & Stelzer, E. H. (2007). The third dimension bridges the gap between cell culture and live tissue. *Nat. Rev. Mol. Cell Biol.* **8,** 839-845.

Petersen, O. W., Lind Nielsen, H., Gudjonsson, T., Villadsen, R., Ronnov-Jessen, L. & Bissell, M. J. (2001). The plasticity of human breast carcinoma cells is more than epithelial to mesenchymal conversion. *Breast Cancer Res.* **3,** 213-7.

Plieva, F. M., Galaev, I. Y., Noppe, W. & Mattiasson, B. (2008a). Cryogel applications in microbiology. *Trends Microbiol.* **16,** 543-51.

Plieva, F. M., Oknianska, A., Degerman, E. & Mattiasson, B. (2008b). Macroporous

gel particles as robust macroporous matrices for cell immobilization. *Biotechnol. J.* **3,** 410-7.

Podrygajlo, G., Tegenge, M. A., Gierse, A., Paquet-Durand, F., Tan, S., Bicker, G. & Stern, M. (2009). Cellular phenotypes of human model neurons (NT2) after differentiation in aggregate culture. *Cell Tissue Res.* **336,** 439-52.

Prestwich, G. D., Liu, Y., Yu, B., Shu, X. Z. & Scott, A. (2007). 3-D culture in synthetic extracellular matrices: new tissue models for drug toxicology and cancer drug discovery. *Adv Enzyme Regul.* **47,** 196-207.

Radisky, D., Hagios, C. & Bissell, M. J. (2001). Tumors are unique organs defined by abnormal signaling and context. *Semin. Cancer Biol.* **11,** 87-95.

Rausch, O. (2006). High content cellular screening. *Curr Opin. Chem. Biol.* **10,** 316-20.

Reininger-Mack, A., Thielecke, H. & Robitzki, A. A. (2002). 3D-biohybrid systems: applications in drug screening. *Trends Biotechnol.* **20,** 56-61.

Rhee, S. & Grinnell, F. (2007). Fibroblast mechanics in 3D collagen matrices. *Adv. Drug Deliv. Rev.* **59,** 1299-305.

Rhee, S., Jiang, H., Ho, C. H. & Grinnell, F. (2007). Microtubule function in fibroblast spreading is modulated according to the tension state of cell-matrix interactions. *Proc. Natl. Acad. Sci. USA* **104,** 5425-30.

Rieke, M., Gottwald, E., Weibezahn, K. F. & Layer, P. G. (2008). Tissue reconstruction in 3D-spheroids from rodent retina in a motion-free, bioreactor-based microstructure. *Lab Chip* **8,** 2206-13.

Semino, C. E., Merok, J. R., Crane, G. G., Panagiotakos, G. & Zhang, S. (2003). Functional differentiation of hepatocyte-like spheroid structures from putative liver progenitor cells in three-dimensional peptide scaffolds. *Differentiation* **71,** 262-70.

Serban, M. A., Scott, A. & Prestwich, G. D. (2008). Use of hyaluronan-derived hydrogels for three-dimensional cell culture and tumor xenografts. *Curr. Protoc. Cell Biol.* **Chapter 10,** Unit 10 14.

Stein, A. M., Demuth, T., Mobley, D., Berens, M. & Sander, L. M. (2007). A mathematical model of glioblastoma tumor spheroid invasion in a three-dimensional in vitro experiment. *Biophys. J.* **92,** 356-365.

Sun, T., Jackson, S., Haycock, J. W. & MacNeil, S. (2006). Culture of skin cells in 3D rather than 2D improves their ability to survive exposure to cytotoxic agents. *J. Biotechnol.* **122,** 372-81.

Sutherland, R. M. (1988). Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science* **240,** 177-184.

Sutherland, R. M., McCredie, J. A. & Inch, W. R. (1971). Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. *J. Natl. Cancer Inst.* **46,** 113-120.

Tabatabai, M., Williams, D. K. & Bursac, Z. (2005). Hyperbolastic growth models: theory and application. *Theor. Biol. Med. Model.* **2,** 14.

Thielecke, H., Mack, A. & Robitzki, A. (2001). Biohybrid microarrays--impedimetric biosensors with 3D in vitro tissues for toxicological and biomedical screening. *Fresenius J. Anal. Chem.* **369,** 23-9.

Timmins, N. E., Dietmair, S. & Nielsen, L. K. (2004). Hanging-drop multicellular spheroids as a model of tumour angiogenesis. *Angiogenesis* **7,** 97-103.

Ulijn, R. V., Jayawarna, V., Smith, A. & Gough, J. E. (2007). Hydrogel compositions. US Patent App. 11/470,962, 2006.

Uriel, S., Labay, E., Francis-Sedlak, M., Moya, M. L., Weichselbaum, R. R., Ervin, N., Cankova, Z. & Brey, E. M. (2008). Extraction and Assembly of Tissue-Derived Gels for Cell Culture and Tissue Engineering. *Tissue Eng. Part C Methods*.

Uroukov, I. S. & Patton, D. (2008). Optimizing environmental scanning electron microscopy of spheroidal reaggregated neuronal cultures. *Microsc. Res. Tech.* **71,** 792-801.

Wang, T. Y., Sen, A., Behie, L. A. & Kallos, M. S. (2006). Dynamic behavior of cells within neurospheres in expanding populations of neural precursors. *Brain Res.* **1107,** 82-96.

Xu, J., Ma, M. & Purcell, W. M. (2003a). Biochemical and functional changes of rat liver spheroids during spheroid formation and maintenance in culture: II. nitric oxide synthesis and related changes. *J. Cell Biochem.* **90,** 1176-85.

Xu, J., Ma, M. & Purcell, W. M. (2003b). Characterisation of some cytotoxic endpoints using rat liver and HepG2 spheroids as in vitro models and their application in hepatotoxicity studies. I. Glucose metabolism and enzyme release as cytotoxic markers. *Toxicol. Appl. Pharmacol.* **189,** 100-11.

Xu, J., Ma, M. & Purcell, W. M. (2003c). Characterisation of some cytotoxic endpoints using rat liver and HepG2 spheroids as in vitro models and their application in hepatotoxicity studies. II. Spheroid cell spreading inhibition as a new cytotoxic marker. *Toxicol. Appl. Pharmacol.* **189,** 112-9.

Zhang, S., Gelain, F. & Zhao, X. (2005). Designer self-assembling peptide nanofiber scaffolds for 3D tissue cell cultures. *Semin. Cancer Biol.* **15,** 413-420.

Zhang, S., Lockshin, C., Rich, A. & Holmes, T. (1997). Stable macroscopic membranes formed by self-assembly of amphiphilic peptides and uses therefore. US Patent 5,670,483, 1997.