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REVIEW

# Modelling tissues in 3D: the next future of pharmaco-toxicology and food research?

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**Abstract** The development and validation of reliable in vitro methods alternative to conventional in vivo studies in experimental animals is a well-recognised priority in the fields of pharmaco-toxicology and food research. Conventional studies based on two-dimensional (2-D) cell monolayers have demonstrated their significant limitations: the chemically and spatially defined three-dimensional (3-D) network of extracellular matrix components, cell-to-cell and cell-to-matrix interactions that governs differentiation, proliferation and function of cells in vivo is, in fact, lost under the simplified 2-D condition. Being able to reproduce specific tissue-like structures and to mimic functions and responses of real tissues in a way that is more physiologically relevant than what can be achieved through traditional 2-D cell monolayers, 3-D cell culture represents a potential bridge to cover the gap between animal models and human studies. This article addresses the significance and the potential of 3-D in vitro systems to improve the predictive value of cell-based assays for safety and risk assessment studies and for new drugs development and testing. The crucial role of tissue engineering and of the new microscale technologies for improving and optimising these models, as well as the necessity of developing new protocols and analytical methods for their full exploitation, will be also discussed.

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**Keywords** 3-D in vitro models · Microenvironment · Pharmaco-toxicology · Food research · Rotating Wall Vessel bioreactors · Microgravity · Tissue engineering

**Abbreviations**

| | |
|---|---|
| ECM | Extracellular matrix |
| 3-D | Three-dimensional |
| 2-D | Two-dimensional |
| RWV | Rotating Wall Vessel |

## Introduction

The need of reliable, human-derived in vitro models alternative to the traditional animal-based studies is increasingly becoming an imperative in basic research and in the more complex fields of safety and risk assessment, as also clearly demonstrated by the new European Chemicals Legislation [53, 71]. Apart from obvious economic and ethical considerations, in vivo animal models are, in fact, progressively showing their limits: although they can mirror many aspects of human responses, they fail to reproduce others. Many pathogens are, for example, species specific (e.g. hepatitis C virus), and it is well known how a leading cause for the failure of new drugs in clinical trials is liver toxicity, which was not predicted by experimental animals [80]. Interspecies differences in metabolism and responses to regulatory signals have also raised questions about the relevance of "humanised" or transgenic animal models in predicting the behaviour of human tissues in vivo [41, 70].

Validated human-derived in vitro test models will be also of extreme value for the early prediction of nutrients

quality and potential health effects of food and dietary supplements. The need to develop appropriate and standardised methods for the analysis of food components efficacy and safety for human health is, in fact, a widely recognised priority. Examples include bioactive peptides [29], botanical products [16] and vitamins [5]. At the same time, food industry could benefit from reliable in vitro tests that can predict in vivo adverse effects of potentially toxic contaminants (i.e. heavy metals, persistent organic pollutants, hormones, chlorination by-products, pesticides and fertilisers), or that can confirm the efficacy of methods adopted to reduce their undesirable effects. Pre-market safety and nutritional assessment procedures for genetically modified plant derived food and feed could also be improved by relevant in vitro/in vivo complementary risk approach strategies, as recently recommended by the European Food Safety Authority (EFSA; http://www.efsa.europa.eu) [22].

Finally, in the field of pharmacology, predictive cell-based assays are expected to improve the success rate at early stages of the drug-discovery process by providing cell-specific responses, which are missing in the target-oriented approach [14]. Moreover, reliable human-derived in vitro models are needed for preclinical "safety pharmacology", as outlined in ICH S7A and S7B guidance documents as well (ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; http://www.ich.org) [92].

Over 2 decades of research have demonstrated that, with respect to traditional two-dimensional (2-D) cell culture systems, three-dimensional (3-D) cell models have the potential to improve the physiological relevance of cell-based assays and to advance the quantitative modelling of biological systems from cells to living organisms [64]. For example, primary hepatocytes become undifferentiated and die within few days if cultured as 2-D monolayer; remarkably, the biosynthesis of drug metabolising enzymes, essential for the toxicity testing in pharmaceutical research, is among the first liver-specific functions to be lost [30]. Re-establishing at least some aspects of the original 3-D microenvironment allows the preservation of hepatic-specific functions for longer periods [69, 79, Mazzoleni and Steimberg, in preparation].

The rapid progress in tissue engineering and in emerging biotechnologies has enormously contributed to generate and optimise innovative 3-D cell-based models. Nevertheless, even if more physiologically relevant 3-D in vitro systems have been developed and validated in recent years for basic research purposes, we still are far from a real strategic application of the related emerging knowledge and new technologies to safety studies, risk assessment and drug discovery fields [7]. Improvements in methods and adaptation to these advances are now required to fully exploit the benefits of the third dimension in all the fields of life sciences.

## Beyond the monolayer

### Limitations of traditional 2-D cell monolayers

To reproduce the phenotype of the target tissue in cultured cells is essential for obtaining reliable biomedical information [6]. Data accumulated over the past 30 years have demonstrated the significant limitations of traditional 2-D cell monolayers in predicting the behaviour of cells in living organisms; nevertheless, due to the fact that they are easy and convenient to set up, they still represent the most popular models for in vitro studies. A major limitation of 2-D monolayers is that they cannot capture the relevant complexity of the in vivo microenvironment. In fact, even if some cell types, such as epithelia, when properly cultured on flat substrates, may exhibit, at least to some extent, differentiated histoarchitecture and functions [54, 85], most cells require cues from a real 3-D environment in order to form physiologically relevant tissue-like structures in vitro [19]. Tissue-specific architecture, mechanical and biochemical signals, and cell–cell communication are lost, in fact, under the simplified 2-D conditions. Moreover, 2-D culture substrates not only fall short of reproducing the complex and dynamic environments of in vivo tissues, but also are likely to misrepresent findings to some degree by forcing cells to adapt to an artificial, flat and rigid surface.

### The third dimension

In vivo, cells develop and grow within the 3-D architecture of tissues and organs. Basing on this consideration, the importance of a 3-D microenvironment in designing physiologically relevant in vitro models of living tissues has been proposed since the 1970s [9, 24, 36, 39], although the relevance of spatiotemporal cellular context during morphogenesis was already recognised by developmental biologists in the very early years of the 20th century [21].

Insights into the different properties of cells cultured in 3-D versus 2-D microenvironments were mainly made by cancer researches. Pioneers in this field, Bissell and colleagues first showed how extracellular matrix (ECM) and tissue architecture shape the way by which normal and malignant cells receive and respond to signals from the surrounding environment [12, 72, 90]. Further studies demonstrated how microenvironmental factors profoundly influence the behaviour of tumour cells, also conditioning their response/sensitivity to therapeutic agents [45, 74, 91, 93].

The large body of studies that have been conducted up to now on cells of various origin has clearly proved the great difference in cell function and behaviour between 2-D and 3-D culture conditions [11, 19, 57, 60, 88, 94, 95]. Also nuclear structure [52], signal transduction [32, 49, 76] and gene expression [10, 15, 28, 42] are quite dissimilar when the same cell type is cultured in 3-D models or in conventional 2-D monolayers. In 3-D systems cells develop into tissue-like structures, more similar to those formed in living organisms [11, 33, 61, 84]. Since they also demonstrated to best reproduce in vivo-like responses [8, 32, 76], 3-D systems have started to be used in a broad range of cell biology studies, including neuroscience [21], tumour biology and morphogenesis [45, 94], while their potential utility for the study of microbial pathogens-host interactions and their possible exploitation in drug development and in innovative approaches for cancer treatment has also been recently emphasised [2, 26, 59].

### Microenvironment specificity and heterotypic cell interactions

While a 3-D microenvironment provides the best lifelike in vitro condition for cell and tissue culturing, other factors, such as the physical properties (stiffness) and the molecular composition of the ECM, were also demonstrated to be important regulators of the cellular behaviour and responses [32, 65–67, 86]. In addition, specific chemical morphogens, growth factors, chemokines and hormones are needed to reproduce the physiological (or pathological) complexity of an in vivo context. A 3-D matrix may both affect solute diffusion and bind many effector compounds, thereby establishing solute tissue-scale and local concentration gradients that are essential to induce cell morphogenesis and functions (see, for example [73]).

The ECM, thus, contributes to the microenvironment specificity not only through its mechanical features, but also through its own signalling moieties and its ability to bind growth factors, enzymes and other diffusible molecules. Cell–ECM interactions are therefore of pivotal importance for normal cell differentiating and functioning, but it is important to take also into account that, physiologically, a given tissue comprises multiple cell populations, that, by interacting with each other, as well as with the shared ECM, lead to unique responses in vivo. Heterotypic cell-cell interactions and the reciprocal effect of different cell populations on the whole microenvironment should be hence carefully considered in trying to establish physiologically relevant in vitro models of tissues/organs.

Ideally, seen the considerations above, each organ and tissue should require its own in vitro specific model, that should comprise a hierarchical arrangement of cells organised within a precisely defined stroma, also inclusive of microvascular networks and proper soluble factors [33].

## Toward tissue-like cell systems

### The evolution of 3-D models

Over the last decade 3-D culture methods have greatly increased in number, due to the rapid advances in culturing techniques emerging from the field of tissue engineering.

At the beginning, cell assembly in 3-D in vitro models resulted in the formation of multicellular spheroids, grown in suspension or on artificial substrates. Homotypic spheroids (comprised of one cell type only) showed a more "physiological" level of cell differentiation in respect to conventional 2-D culture conditions [37]. Heterotypic spheroids (composed of different cell types from the same tissue) were the first step towards the in vitro reconstruction of complex 3-D tissue equivalents. Even if they provided new insights into our knowledge of multicellular responses to physical or chemical injury [43, 77], or to the process of tumorigenesis [48, 83], the presence of a central hypoxic area that undergoes necrosis strongly limited their use. Central necrosis, due to mass transport restriction, toxic metabolites accumulation and lack of nutrient penetration, was also the limiting factor in the culture of organ explants. The increasing importance given to the physicochemical properties of tissue-specific microenvironments, led then many research groups to develop monotypic cell models that, in addition to cell source and medium components, carefully considered the extracellular matrix as a key element to support tissue-specific differentiated cell functions, as well. Collagen type I, fibronectin-/laminin-rich basement membrane substitutes (e.g. Matrigel), reconstituted basement membranes, 3-D collagen gels, natural cell-derived 3-D extracellular matrices or fully synthetic polymeric 3-D nanostructured microenvironments have been extensively used (for a recent review on the subject, see [50]), and, even if they have added new and valuable insights to the understanding of cell functions in 3-D microenvironments (e.g. cell adhesion and migration, polarity, branching morphogenesis), yet they have not provided clear correspondence with the in vivo counterpart, whereas, unequivocally, they demonstrated the importance of matching cell types with appropriate substrata ([32, 76, 94] and references therein).

Since, beside specific matrices and other environmental factors, organs comprise multiple cell types, organotypic co-cultures that best approximate the whole tissue/organ environment have been then developed. "Skin equivalents" represent the most diffused and successful 3-D organotypic models that, effectively, have been

productively used in pharmaco-toxicological studies (e.g. [89]) or for grafting procedures [13]. So far, other epithelial tissues have been successfully modelled in 3-D ECM cultures (see, for example [34, 95]), and heterologous 3-D organotypic cultures have been extensively used in order to study interactions between different cell types in normal and pathological conditions ([45, 67, 68] and references therein), to simulate in vitro human malignant tumours [25, 81], to investigate factors that regulate stem cell fate [17], and to learn about virus-host interactions [2].

Table 1 lists the principal 3-D cell culture systems currently employed in basic and applied research.

## Increasing mass transfer

### *Bioreactors*

It is well known that the metabolic requirements of 3-D cell constructs are substantially higher than those of flat cell monolayers, grown in static environments in liquid media.

Bioreactors were primarily developed to modulate mass transfer, a crucial element for guaranteeing gas/nutrient supply and waste elimination, essential factors for maintaining cell viability within large 3-D cell/tissue masses. Mammalian tissues are among the most difficult tissues to

**Table 1** Summary of the principal currently available 3-D culture systems

| System | | Advantages | Disadvantages |
|---|---|---|---|
| ***Whole perfused organ*** ***( "ex vivo" model )*** | | – Complex 3-D arrangement of cell–cell and cell–matrix interactions is preserved<br>– Closest to the *in vivo* situation | – Technically complex<br>– Need efficient perfusion systems (mass transfer and waste elimination is variable throughout the organ)<br>– Cell viability is limited to few hours |
| ***Tissue slices / Tissue explants*** | | – Preserve part of tissue architecture and cellular interactions<br>– Maintain tissue-specific functions for longer time than perfused organ | – Cell viability and differentiated phenotype are limited to few days |
| ***Scaffold- / Microcarriers-based cultures*** | – ***Naturally derived materials*** (e.g. collagen or fibrin)<br>– ***Synthetic polymers*** [e.g. poly(dimethylsiloxan), poly(DL-lactide-co-glycolide), poly(glycerolsebacate)] | – Engineered scaffolds provide physical/ structural/ biochemical support<br>– Sustain cell viability and tissue-like functions<br>– Can be used in all dynamic/ perfused culture systems | – Spatial variations in nutrients, oxygen, metabolite concentrations may exist and modify cell behaviour randomly throughout the scaffold |
| ***Organotypic cultures*** | ***Multicellular spheroids*** | – Reconstitution of tissue-like organisation (polarity, function, viability)<br>– Scaffolds can be avoided | – Cell life span may vary, depending on the type of bioreactor |
| | ***Cell sheet engineering***[a] | | |
| ***Gel- / matrix-based cultures*** | ***Hydrogels (ECM like):***<br>• *Natural product-based hydrogels* (e.g. collagen, fibrin, Matrigel)<br>• *Synthetic self-assembling peptide hydrogels* | – Differentiated phenotype can be maintained for several days, depending on the cell type | – Harvesting of cells could be optimised<br>– Problems of mass transfer if not coupled to perfused systems |
| | ***3-D surfaces (BM like):***<br>• *Synthetic* (fixed combination of various ECM components)<br>• *Cell-/ tissue-derived biomatrices* | – Situation more similar to *in vivo* conditions<br>– Sustain cell viability, polarity, function | – Composition and structure of matrices may vary between preparations<br>– Standardised protocols are needed |
| ***Cell suspension*** | | – Easiest to handle of all *in vitro* models | – Very limited lifespan (for hepatocytes: 2 to 4 hours) |

*ECM* extracellular matrix, *BM* basal membrane

[a] Layers of cells cultured on top of porous membranes/surfaces

keep in vitro, due to their important and specific nutrient needs, their sensitivity to nitrogenated wastes, and their fragility to shear stress [27]. Differences between tissues also require specific culture characteristics that have to be taken into account. Oxygen tension (defect or excess) is also a limiting factor in the in vitro culture of cell/tissue constructs. A specific bioreactor configuration (design and operational conditions) should then be based on the precise evaluation of all these parameters (extensively discussed by [55]).

The first-generation bioreactors were designed to maintain 2-D tissue constructs, such as skin [31], or tissue sheets/patches [20, 82] for clinical applications. The dynamic culture conditions initially obtained by stirred tank bioreactors, were, effectively, helpful in favouring mass transport at greater depth level within the 3-D cell constructs, if compared to the conventional static environment of liquid overlay techniques. Tissue engineering gave then, with its interdisciplinary approaches, new opportunities for trying to generate 3-D mammalian cell constructs able to maintain in vitro tissue-specific differentiated functions in dynamic fluid microenvironments: complex extracellular matrices, microcarriers or scaffolds of various materials were used for promoting 3-D cell assembly and survival in spinner flasks, roller tubes and gyratory shakers. Even if hydrodynamic forces effectively increase mass transfer, nevertheless, larger cell aggregates (exceeding 1 mm in size) and tissue explants cultured in vitro in these experimental conditions still developed necrotic cores; moreover, a major restriction of these 3-D culture models was the detrimental effect of the high shear stress on cell viability and differentiation.

From orbitally mixed Petri dishes, to continuous stirred-tank reactors, up to the more sophisticated hollow fiber (HF), coaxial HF and multi-coaxial HF, Couette-Taylor or airlift bioreactors, the technological progress allowed to improve further oxygen and nutrients supply to cultured cell/tissue structures; however, even in the case of the recently developed packed-bed bioreactors, these devices still do not allow to generate optimal conditions for the long-term culture of functional tissue-like masses (for detailed reviews on the subject see [55, 58]). The current generation of bioreactors has, in fact, been developed to support the rapid growth of cells in solution and not the culturing of tissue engineered constructs [38].

### *Culturing cells in microgravity: the Rotating Bioreactors*

Low-shear environment and optimal mass transfer were attained with the introduction of the Rotating Wall Vessel (RWV) bioreactors. Fruit of N.A.S.A.'s Johnson Space Center technological research in USA (http://science.nasa.gov/NEWHOME/br/bioreactor.htm), and used in ground- as well as in space-based studies on a wide variety of cell types and tissues, the RWV devices are commercially available from Synthecon Inc. (Houston, USA). With no internal moving parts, the RWV bioreactors are horizontally rotating, transparent clinostats, that leave no head space between atmosphere and culture medium, therefore reducing shear forces and turbulence normally associated with impeller-driven stirred bioreactors, to a minimum; sedimentation and inadequate gas/nutrients supply are also avoided, thus guaranteeing the most favourable conditions for cell/tissue culturing [78].

RWV clinostats are equipped with a culture chamber that rotates around a horizontal axis, so that, by adjusting its rotational speed according to the specific experimental needs (e.g. sample number, density or dimensions), it is possible to obtain a stable condition where the gravitational field is time-averaged to near zero over each revolution (vector-averaged gravity), thus, effectively, negating the influence of gravitational sedimentation (balanced with centrifugation and fluid drag) and reproducing some aspects of microgravity (simulated microgravity) [46]. An optimal diffusion of gas (oxygen) inside the culture chamber is ensured by an efficient gas exchange membrane; continuous monitoring of gas supply, pH and temperature for culture periods ranging from several days to many months is also allowed. The RWV operational conditions can be constantly monitored and optimised during all the experimental procedures, in order to obtain a laminar flow of the fluid medium inside the culture chamber, thus reducing to a minimum the mechanical stress (shear force) on the biological samples surface; the rotational speed of the chamber can be continuously controlled, so that the samples (non-rotationally stabilized) remain in a constant orientation with respect to the chamber wall, and move in a near-solid body rotation with the fluid, thus fulfilling the requirements needed to successfully simulate microgravity condition [3]. This simulated microgravity condition facilitates space co-localisation and 3-D assembling of large cell aggregates; randomised gravitational vectors may also promote cell aggregation and differentiation processes by directly affecting gene expression or, indirectly, by facilitating autocrine/paracrine cell interactions [40].

Tissue-like 3-D constructs, as well as many different cell types from various origin and intact tissue explants, have been demonstrated to be kept efficiently in culture by the RWV bioreactors, even for long periods ([35, 59, 87]; see also Synthecon web site at http://www.synthecon.com for the latest information on the different cell types/tissues that have been successfully cultured in RWVs).

The unique microenvironment generated by the RWV bioreactors thus provide an excellent in vitro system for evaluating cell–cell and cell–matrix specific interactions, as well as for testing the influence that physical or chemical

factors may have on cell behaviour. The utility of these devices in generating 3-D cell models for the study of human infectious disease has also been recently highlighted [59].

Synthecon also developed a spherical reactor, in which the flow conditions are non-linear [1], and a Perfused Culture System (Rotary Perfusion RCMW), that allows inline monitoring of pH, oxygen and glucose levels and where medium can be exchanged, sampled, or modified without stopping the vessel rotation (Fig. 1); both of these devices are currently under evaluation.

## Tissue engineering and microtechnologies: the perspectives

The multidisciplinary field of tissue engineering is crucial for the development of new in vitro models that can be tailored for specific applications. First, basing on quantitative analyses of cell and tissue behaviour, it can provide new information on regulatory chemical or physical signals that govern cell–cell and cell–environment responses, and, secondarily, it can develop and supply a toolbox of biomaterials, scaffolds and devices that allow the formation/maintenance/study of accessible 3-D functional tissue structures in vitro [33]. Microscale technologies are emerging powerful tools for tissue engineering that can help in generating physiologically relevant, reproducible and well controlled cell-based systems [44]. Microfabrication techniques allowed the development of a wide range of synthetic nanostructured 3-D substrates, now available for cell culturing, that are promising for ensuring more reliable and specific 3-D microenvironments for cell models [75]. Coupling microfabrication of physically and chemically defined 3-D surfaces/scaffolds with advanced photo patterning, soft lithography techniques and microfluidics has led to a great enhancement in the complexity and biomimetic properties of engineered cell constructs [44]. Even if further significant research and technological progress is needed, the possibility to integrate these systems with devices for multiple and simultaneous microscale analysis of cell behaviour and responses, will open concrete opportunities for realising, in a next future, multifunctional 3-D cell-based platforms for a large number of applications, including screening tests [23, 56]. Cell/tissue culture units coupled with biosensors should be used, for example, for on-line automated monitoring of environmental (e.g. gas tension, pH, temperature) and cell-linked (e.g. growth rate, density, metabolism) parameters.

Examples of the application of these new technologies that may, potentially, provide promising tools for generating adequate and cost-effective in vitro models for basic research purposes and pharmaco-toxicological needs, have been recently reported. An innovative 3-D model of human oral carcinoma, based on the use of a highly porous polymeric scaffold, was, for example, developed and tested for drug responsiveness by Fishbach et al. [25], while Ohashi et al. [63] engineered an uniformly continuous sheet of hepatic tissue, that shows liver-specific functions, using isolated primary hepatocytes cultured on temperature-responsive surfaces. 3-D cell cultures have been also successfully used as screening tools for microscale toxicology assays [51], while Robitzki and colleagues generated and tested a 3-D multifunctional electrode-based microcavity array, which can be used for impedance spectroscopy to analyse in 3-D multicellular cultures (spheroids) cell-type specific responses to chemically active compounds [47] .

Computational fluid modelling will be a powerful tool as well, that, by contributing to generating and optimising

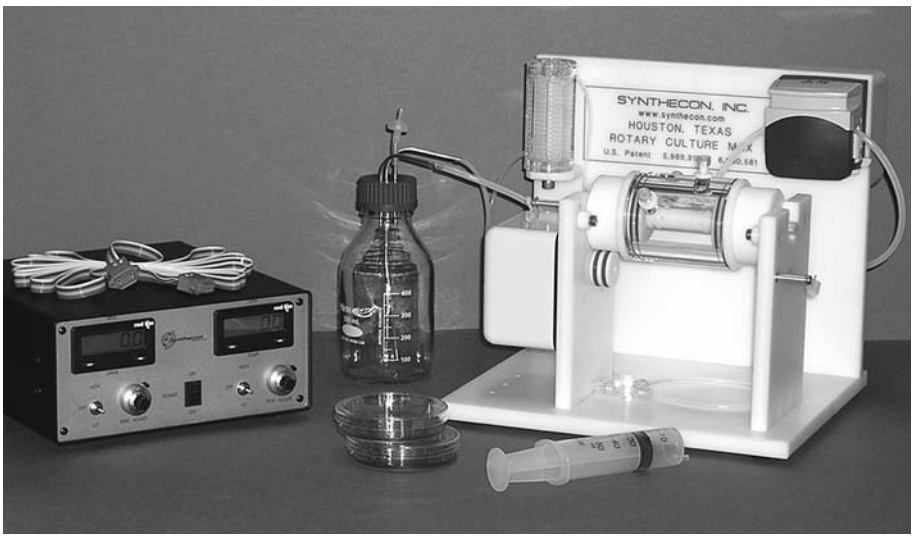


**Fig. 1** The Perfused Culture System (Rotary Perfusion RCMW)

tissue engineering-related bioreactors, can be helpful in reproducing specific physiological environments for 3-D cell constructs [38]. Model systems made of different compartments configured for representing the various individual tissues/organs (functional tissue equivalents) could be then generated and hierarchically connected by controlled medium exchanges, designed to reproduce the metabolic interactions that physiologically take place between organs, thus simulating the situation of a whole living organism. To develop bioreactor systems able to mimic the complexity of the metabolism of a living organism, especially in the case of humans, represents a challenge, and, if successful, can not only be a real alternative to the use of experimental animals, but can, potentially, provide new knowledge of human metabolic processes and responses to various chemical/physical stimuli.

In summary, the most commonly used approaches currently available to generate 3-D models, in static or in dynamic conditions, can be schematically grouped as follows: (1) organotypic explant cultures, generally kept on a substrate in the presence of media; (2) scaffold-/microcarriers-based cultures; (3) micromass cultures (homo-/hetero-typic spheroids); (4) gel-/matrix-based techniques; (5) microfluidic systems. Table 2 illustrates advantages and disadvantages of the most commonly used static and dynamic 3-D culture systems.

## What else is needed

Together with all the technological innovations in new materials and devices expected from tissue engineering, the use of 3-D culture models will also require substantial innovation in methods (e.g. of sample handling and analysis), as well as in imaging techniques [64]. Among the experimental and technical improvements that are needed to take full advantage from 3-D cell biology, a systematic collection of methods that help scientists in the transition from monolayer culture to the 3-D systems is, in fact, still lacking [33]. Reliable and standardised methods of long-term in vitro culturing of organ explants slices, that, even if depending on the need of constant supply of living tissues, have the advantage to preserve the original cytoarchitecture and the cellular differentiation of the original native tissues, thus permitting to provide tissue-specific information to a level of complexity closer to that of the intact organism, need to be optimised and validated. 3-D cell culture protocols also need to be improved and standardised, according to the biological applications they are directed at. New and more suitable methods have to be established for the analysis of 3-D systems; for example, new parameters should be defined to guarantee optimal control of culture conditions, as well as new protocols should be optimised to separate cells from the matrix to perform

**Table 2** Static and dynamic systems currently employed for 3-D culture

| System | | Advantages | Disadvantages |
|---|---|---|---|
| ***Static systems*** | – Liquid overlay cultures<br>– Static matrix cultures | – No shear stress | – Limited mass transfer<br>– Low cost<br>– Limited cell survival |
| ***Dynamic systems*** | – Roller bottles<br>– Gyratory shakers<br>– Spinner flasks | – Moderate to high mass transfer to provide nutrients and to export wastes<br>– Increase cell viability and allow long-term studies | – Intermediate to high shear stress<br>– More or less expensive, depending on the system in use<br>– Difficult to use at large scale |
| ***Rotary cell culture systems*** | – Rotary Cell Culture Systems (RCCS) | – Low shear forces and turbulence<br>– High mass transfer<br>– Maintain and/or favour tissue-like organisation, polarity, function<br>– Increase cell viability | – Expensive |
| | – Rotary Perfusion RCMW | – Perfusion extends RCCS potential<br>– Available as multi-compartmental devices | – Difficult to handle for screening test purposes<br>– Expensive |
| ***Perfused models*** | – Hollow-fiber perfused systems<br>– Airlift bioreactors<br>– Direct perfusion bioreactors<br>– Packed-bed bioreactors | – Controllable and reproducible microenvironment<br>– Low to intermediate shear stress<br>– Efficient mass transfer<br>– Long-term maintenance of tissue-like functions and cell viability<br>– Work with multi-compartmental devices allowed | – Limited 3-D cell growth<br>– Cost more or less significant, depending on bioreactor type |
| ***Microfluidic systems*** | – Microfluidic systems<br>– Microfluidic biochips | – See Perfused models<br>– Nutrient / fluid gradient possible<br>– Lab on chip (on-line investigations) | – Limited 3-D cell growth |

biochemical analyses [62]. Imaging techniques that, considering the complexity of the requirements that 3-D living specimens present, can at the same time penetrate such thick (and, sometimes, scattering) samples with minimal damage and high spatial resolution, allowing, for instance, dynamic quantitative analyses of such structures, have also to be developed (discussed by [64]).

## Conclusions

Physiologically relevant models for the study of normal cell/tissue functions and disease progression, as well as for the development of new therapeutic strategies or predictive toxicological investigations, should take into account that organs and tissues function in a 3-D environment, where extracellular matrix, homo- and hetero-typic cell interactions, and various biochemical and biophysical factors greatly condition cell specificity; they should also recognise that the organ itself is the unit of function. Nevertheless, a hierarchy of related models should exist that, even recognising the importance of clearly defined 3-D microenvironments, can vary with respect to their complexity to be suitable and accessible either for basic research (i.e. for identifying molecular determinants of normal organ functions and for elucidating pathways compromised during disease progression), or for pharmaco-toxicological applications (drug design and testing) [76]. In this context, the implementation of high-throughput screening procedures based on the use of simpler 3-D organotypic models, will be of great value [4, 6, 48].

Microgravity-derived 3-D cell constructs and tissue engineering may provide, for the future, useful tools for generating reliable model systems that couple cheapness and handiness with an increased predictive power, also fostering the integration process of data from genomics, proteomics, metabolic profiling and molecular cell biology [64].

"Exploiting the third dimension" is a big challenge for the next decades in life sciences [64], and will require a more complete integration of systems biology approaches into the design and analysis of engineered tissues [18]. The rapid progress in the development of the new micro- and nano-technologies, together with the improvements in imaging technologies and in the establishment of standard experimental protocols, will certainly enhance the possibility of a rapid advance toward this objective.

Integrated and intelligent strategies involving in silico and sophisticated in vitro procedures will provide new and more efficient approaches in safety pharmacology, drug discovery and toxicity testing, as well as in all fields of food research, with consequent direct benefits for human health and undoubtable advantages in terms of industrial efficiency and animal welfare; this will be possible only if proper effort is invested in these fields, which should also be sufficiently adaptable to this change [7].

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