Three-dimensional (3D) models

Tri-dimensional (3D) culture models, such as organoids/spheroids and organ-on-chips, are gaining traction as relevant tools for biomedical research. Their use has grown exponentially in the past decade (62 indexed publications in PubMed in 2011 vs 2,948 in 2021). Even though organoids are far from replacing animal experimentation, they can eventually help abolishing the use of animal models in certain areas. Furthermore, like *in silico* approaches and *in vitro* cell culture techniques, they are an invaluable tool to considerably reduce the use of *in vivo* experimental models.

3D culture models can help filling the gap between simple cell monolayer cultures and the intricate complexity of a fully developed organism. These techniques are better models than immortalized cell lines since they are genetically more stable, contain a more heterogeneous population and develop complex morphologies (Aguilar, 2021). Among this heterogeneous population, there are progenitor cells that can differentiate into various cell types; hence organoids roughly adopt the architecture of the tissue they derive from and can maintain this phenotype through multiple passaging. Owing to this progenitor cells, organoids are suitable for very long culture times. That is why organoids are the pillar of 3D cell cultures.

The culture conditions of 3D models often rely on the use of a matrix that simulates the extracellular membrane. These matrices are enriched in glycoproteins (i.e., collagen, laminin, nidogen, etc.) and growth factors. Perhaps the most extensively used reagent for organoid culture is Matrigel® (Corning), but other components, such as Hydrogels and collagen-based matrixes are also available. Owing to their culture conditions within matrix gel droplets or matrix-enriched media, organoids grow in a spherical shape, that is why they are often also called “spheroids”. This spherical architecture can sometimes hinder the study of certain cellular types, especially in luminal organoids (i.e., airway and gastrointestinal organoids) in which the epithelial cells locate inside the spheroid. That is why strategies where 3D models are turned into 2D cultures have been designed. These strategies try to maintain the heterogeneity of cellular populations and even try to mimic other environmental conditions, which is the case for Air-Liquid Interface (ALI) cultures, organ-on-chips, or synthetic organoids.

A) Organoids

As aforementioned, organoids offer the advantage of a complex organ-like culture without the need of using an animal model. Nevertheless, the generation of organoids of either human or animal origin still depends on the obtention of biological samples through invasive procedures.

Generation of organoids and ethical aspects

Organoids can be of diverse origin. The source material must contain progenitor cells that can become terminally differentiated cells. Most frequently, Adult Stem Cells (ASC) are used for this purpose. ASCs can be obtained from biopsies and necropsies. Alternatively, Embryonic Stem Cells (ESC), that have pluripotency, can be differentiated into all types of tissues using the adequate transcription factors and

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UniversitätsKlinikum Heidelberg • Voßstraße 2, Geb. 4040 • 69115 Heidelberg, Germany
• e-mail: transvacinfo@euvaccine.eu

signalling pathways. Finally, the development of induced Pluripotent Stem Cells (iPSC) represents a new source of pluripotent stem cells that bypasses most of the ethical and technical issues related to the use of ESCs.

The generation of animal-derived organoids often implies the euthanasia of the donor animal to obtain fresh tissue (intestinal crypts, airway cells, liver samples, brain progenitors, etc.) (Kar et al., 2021). As long as organoids are used for multiple experiments, this would still comply with the 3R principle of reduction. Furthermore, the obtention of the source material must comply with the 3R principle of refinement.

In comparison, the use of human-derived organoids must consider broader ethical aspects. First, biological samples are often obtained as a by-product of a surgical intervention under a previous informed consent by the patient. This informed consent must deal with a wide range of topics, from the generation of the organoid, biobanking, the intended use for research and/or even potential uses beyond research. Since all these applications are difficult to foresee, the informed consent should be written in a detailed, while comprehensive enough manner, to respect the donor's will.

In comparison, the use of ESCs derived from human embryos generates hesitancy in some countries where they give these cells a moral status. Experimentation with ESCs is accepted in many countries until a certain stage of development. However, a consensus is missing regarding this matter, and in some cases, it is strictly prohibited. This raises the question whether it would be widely accepted to perform experiments with ESC-derived organoids. Alternatively, the use of iPSCs bypasses the mentioned dilemma since these cells are reprogrammed from terminally differentiated cells. Still, their obtention must be properly supported by the informed consent of the donor, and many aspects must be considered. All these bioethical dilemmas and the ethical and moral issues specific to each type of organoid are further discussed by Mollaki, 2021.

Organoids in vaccine development

In the context of vaccine development, 3D cultures have eased the study of cellular pathophysiology, tropism and the host-pathogen interaction of multiple microorganisms.

Despite the increased heterogeneity of cell types in organoids, these still lack the complex interactions between the organ and the diffuse immune system. That is why organoids have been mostly used to study how a specific virus infects a target cell type from the parenchyma, or how these cells display innate immune signatures or cellular re-arrangements upon infection. Several examples of these studies that have a clear impact on vaccine development for infectious diseases can be found in **Annex 2**. Co-culture of organoids with immune cells ($CD4^+$ and $CD8^+$ T cells, NK cells, dendritic cells, and macrophages), or using secondary lymphoid tissues could result in even more relevant models for vaccine development (**Annex 2**).

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B) Organ-on-a-chip

Organ-on-chips are also a promising platform to study diseases in an *in vitro*, 3R compliant platform. They could become a useful tool to model complex environmental culture conditions. They can be generated from primary cells or from organoids and/or immortalized cell lines. The main difference with organoids is that organ-on-chips cannot sustain long culture periods, and they are usually assembled for short-lived experiments.

In this context, chips are often built as a co-culture with endothelial cells (mostly obtained from cord blood) and/or immune cells derived from PBMCs. Other strategies try to simulate mechanical tension or peristaltic movements of the lungs and intestines, respectively, using microfluidics, air-liquid interfaces, or complex biomedical microelectromechanical chips (**Annex 3**). These synthetic organ-on-chips, in some cases, offer more physiological functions that can be relevant for a deeper understanding of host-pathogen interactions and vaccine testing (Tang et al., 2020).

Despite standing as a promising platform to elucidate the intricate interactions in multi-cellular complex models of diseases, organ-on-a-chip approaches are technically demanding, and platforms are expensive. The standardisation of these techniques could help making this technology more accessible for a wide range of researchers, especially those with a strong interest for virological and immunological research.

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