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How to optimize respiratory models for SARS-CoV-2 research

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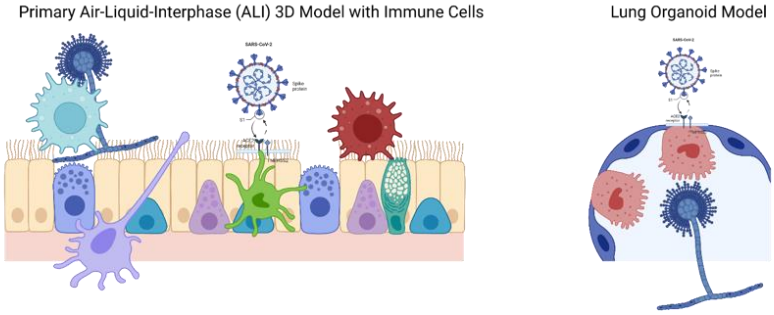
Abstract



Sophisticated 3D cell culture tissue models experienced a boom in the last years and in particular human cell culture and 3D respiratory systems greatly supported the development of novel drugs and vaccines during the SARS-CoV-2 pandemic. These models provide multiple benefits in terms of similarities in differentiation, metabolism, receptor expression, polarity, and infectivity compared to human tissues and thus provide excellent models to study the first interactions with the host during pathogen entry. Depending on the experimental approach, the use of 3D models is beneficial – apical-out lung organoids for high content screening (HCS) of treatment options and air-liquid interphase (ALI) models for easy incorporation of immune cells, screening of epithelial integrity or mucociliary clearance. This review gives an overview on the models established in our laboratory and their applications.

1. Introduction

The use of organotypic three-dimensional (3D) cell cultures in basic, translational, and clinical research is now more popular than ever - rapid development is taking place in this area due to modern technologies, commercial availability of induced pluripotent

stem cells (iPSCs), and more physiologically relevant primary cells or biobanking opportunities.

Often, these novel approaches use self-assembling 3D organoid or spheroid cultures, which offer several advantages over conventional 2D cell cultures. In addition to 3D organoid/spheroid cultures, there are other options for 3D cultivation, such as scaffold-based methods and long-term differentiation within an air-liquid interphase (ALI) for example, as used in respiratory tissues to mimic the situation in the respiratory tract *in vivo*. 3D culture technologies offer great potential for a more realistic disease modeling *in vitro* and for testing drugs in a personalized way since they behave similarly to the human body in terms of topology, gene expression, signaling, and metabolism (Frieboes et al 2006; Ghosh et al 2005; Mazzoleni et al 2009; Pampaloni et al 2007; Semino et al 2003). The simulation of tissues in cell cultures offers the opportunity for answering scientific questions that cannot be met using conventional 2D cell culture monolayers or animal experiments. A 3D model based on human cells offers more complexity compared to 2D culture and is closer to the human system compared to model organisms. Thus, we review here the current progress of respiratory tissue model optimization performed in our lab during the last 10 years and highlight recent findings on pathogen-barrier interactions using a viral (SARS-CoV-2) challenge.

2. Respiratory 3D models and their applications

Depending on the experimental approach, the use of various 3D models is more valuable – e.g., apical-out lung organoids are highly suitable for high content screening (HCS) of therapeutic options, and ALI models for simple incorporation of immune cells, evaluation of epithelial integrity, or mucociliary clearance.

2.1 Lung organoids

Organoids are 3D cell aggregates produced *in vitro* that correspond to differentiated tissues or even 'mini-organs'. Organoids originate in tissues that contain stem cells, allowing them to self-assemble *in vitro*. The organoid field owes its progress in particular to the development of stem cell technologies over the past two decades. They can be generated from adult tissue-specific stem cells or from iPSCs by adding specific growth factors. The organoids spontaneously organize into organ- or tissue-like structures and the cells typical for the tissue or organ are contained in the organoid – thus organoids are heterogeneous with respect to their cell composition in contrast to the mostly homogeneous feeder layer cultures. Thanks to technological advances, adult stem cells can now be cultivated over prolonged periods of time, e.g. adult stem cell organoids from lung (Figure 1), small intestine, liver, skin, and other epithelia (Clevers et al 2016; Li et al 2016; Salahudeen et al 2020; Takahashi et al 2019). Organoid cultures offer a good base to study host pathogen-interactions or perform drug studies (Takahashi et al 2019; Bergdorf et al 2020; Bershteyn et al 2017; Drost et al 2018; Skardal et al 2016). As shown in Figure 1, respiratory organoids express markers that are found *in vivo*, such as ACE2 and TMPRSS2 (Song et al 2020; Baratchian et al 2020; Lukassen et al 2020). The organoid structure largely depends on the ability of the respective cells for self-organization. The size of organoids is usually limited due to restricted diffusion of nutrients and oxygen in living tissue using conventional scaffold-based techniques. Under such conditions, cells in the deeper regions of the organoid die due to hypoxia and nutrient deprivation, hence

organoids offer a good model to study tumors that lack efficient oxygen supply. It is therefore not possible with these technologies to reproduce the anatomy of larger tissues or organs, including vascular networks. In contrast, novel technologies using a vertical, rotating incubator equipped with bioreactors allow generation of large and living organoids. ClinoStar (CelVivo, Denmark) is a specific incubator, in which organoids can be cultured without addition of any scaffold like Geltrex™, VitroGel™, GrowDex™, or similar. The incubator is based on bioreactors equipped with hydration balls. This area is connected to the actual reactor by a membrane, so gas exchange takes place at all times. The single cells are transferred to the reactor in 10 mL medium and placed in the incubator. There is space for up to six bioreactors in the incubator and each reactor can be controlled independently. The bioreactors are in constant rotation, whereby the speed and direction can be adjusted individually. Due to the constant movement, the single cells combine to form organoids. A camera is installed in the incubator for each position, so that the condition of the organoids can be constantly monitored until they are large enough, without having to open the incubator (CelVivo; Wrzesinski et al 2021). Organoid culture still harbors some problems such as missing vascularization or the difficult incorporation of immune cells into the system. Hence, intense research within this field is needed to obtain solutions (Zhao et al 2021; Ziolkowska et al 2021). Nevertheless, organoids are useful for high content/high throughput testing of potential novel drugs and vaccines within a human organ-like structure (Salahudeen et al 2020; Han et al 2021; Rijsbergen et al 2021). Generation of lung organoids and re-polarization into apical-out lung organoids used for infection with SARS-CoV-2 variants (wildtype and variants of concern) were recently illustrated in more detail in studies by Posch et al (2021a, b).

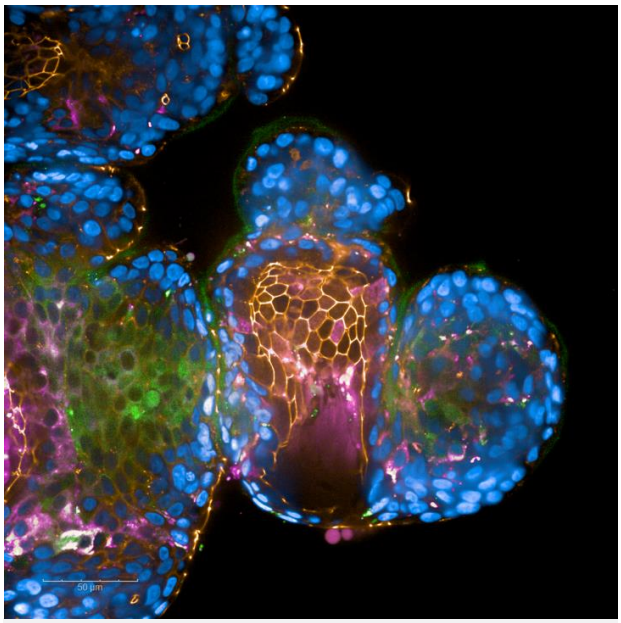


Figure 1. Lung organoids express ACE2 and TMPRSS2. Lung organoids grown in Geltrex™ were stained using the SARS-CoV-2 entry molecules ACE2 (pink) and TMPRSS2 (green), F-actin (Phalloidin, orange) and nuclei (Hoechst, blue).

2.2 Respiratory air-liquid interphase cultures

In vitro approaches to recapitulate human respiratory diseases involve the use of normal human primary epithelial cells of nasal, bronchial, or tracheal origin, typically cultured on biocompatible matrices (cellulose, collagen, alginate, gelatine, elastin, Matrigel®) to mimic the *in vivo* environment (Bovard et al 2018; Ghosh et al 2020; Pharo et al 2020). As a further step to improve the physiological relevance of these models, respiratory primary cells are cultured under ALI. Under these conditions, the cells differentiate into a stratified (pseudostratified) epithelium containing basal cells, ciliated cells, and mucus-producing goblet cells (Figure 2). Complex 3D *in vitro* systems, which contain immune cells in addition to the airway epithelia mentioned above and are

stimulated with airborne particles, are valuable tools for characterizing host-pathogen interactions in tissues of the respiratory tract. Various approaches to design sophisticated *in vitro* systems are currently being developed, but these may lack the immune component. This can be circumvented by designing epithelial/immune cell co-cultures, where immune cells are added to differentiated barrier models of the upper and lower respiratory tract. Such immune/barrier models are particularly well suited to investigate interactions with pathogens or harmful challenges on the respiratory tract, such as bacteria, fungi, cigarette smoke, or SARS-CoV-2, which causes COVID-19. Over the last decade, a respiratory epithelial/immune model was optimized in our lab allowing for perfusion and repeated imaging of the same sample (Zaderer et al 2019). Perfusion was described in detail in Chandorkar et al (2017), introducing relevant immune cells for analyzing epithelial/immune interactions with fungi (Chandorkar et al 2017; Luvanda et al 2021a, b). Here we describe the work by Zaderer et al (2019) in more detail.

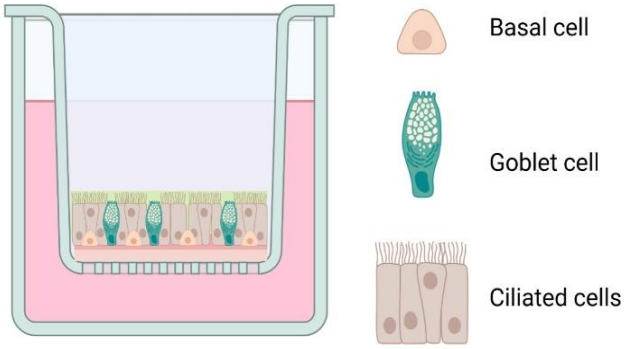


Figure 2. ALI culture of Normal Human Bronchial Epithelial (NHBE) cells. Seeded to Transwells, primary respiratory cells establish a pseudostratified epithelium in about 2 weeks of ALI culture. Within the epithelium different cell types as basal, goblet and ciliated cells occur.

2.3 Immunofluorescence / live cell imaging improvement in ALI cultures

Live cell imaging is an important tool to characterize cellular processes, such as proliferation and differentiation. In ciliated epithelia, live cell analyses are applied for assessing mucociliary clearance that provides a first defense against pathogens attaching to the mucous layer – this mechanism is necessary for full function of the lungs (Chateau et al 2018; Gamm et al 2017; Puchelle et al 2006; Thompson et al 1995). Various protecting molecules ensure aggregation, trapping, and killing of microbes (Whitsett, Alenghat 2015). Extracellular fluid is shifted by one-directional cilia beating towards upper parts of the mammalian airway, clearing the lungs from inhaled pathogens. To allow live cell imaging and monitoring of the same Transwell over time for examining differentiation of cells and mucociliary clearance, we switched the 'world' of the cells and seeded the cells upside-down. This technique allows transfer of the Transwell from the original plastic well plate into a liquid drop within a glass-bottom plate under sterile conditions. The same Transwell can then be analyzed for its differentiation using live cell immunofluorescence and compounds appropriate for the live cell detection of cilia (wheat germ agglutinin), mitochondrial activity (MitoTracker, MitoSOX), nuclei (Höchst), cytoskeleton (CellMask, BacMam 2.0), and mucociliary clearance capacity using fluorescence-labeled beads. By this modified protocol, we found that upside-down seeding of cells within a xeno-free, birch-based cellulose hydrogel (GrowDex™, UPB Biochemicals) exerted positive effects on proliferation and differentiation of primary respiratory epithelial cells. The animal-free cellulose hydrogel comprised a significantly faster differentiation of upper and lower respiratory epithelial cells even under static conditions: cells were fully differentiated after 2 weeks compared to 3 weeks in rat-tail

collagen. Moreover, upside-down seeding within cellulose enabled using the same Transwell inserts over time. In addition, mucociliary clearance can be analyzed in a more realistic setting, using the upside-down seeded cells since the cells are not constricted by plastic barriers from the Transwell chamber, as seen when seeding cells in the normal orientation of the Transwell. Also in upside-down conditions, easy addition of immune cells is feasible by pipetting immune cells into the upper chamber of the insert, while the air side is in the lower chamber. These optimization procedures make the upside-down well a valuable tool for repeated exposure experiments, for live cell imaging over prolonged periods of time, and for monitoring and evaluating mucociliary clearance after infection or treatment with e.g., antiviral sprays (Posch et al 2021c). Thus, the ALI cultures provide more physiologic conditions compared to organoid cultures but are limited to high content testing. Accordingly, the workflow in our lab is to test many antifungal or antiviral compounds in high throughput in apical-out organoids prior to studying the most promising ones in immune-competent ALI cultures. These can be equipped, within a single sample, with more than one immune component by simultaneous addition of autologous macrophages, dendritic cells, NK cells, granulocytes, T and B cells, and humoral compounds, consequently reflecting more realistically the situation in the human body.

2.4 Respiratory 3D models and SARS-CoV-2

Early events following transmission of SARS-CoV-2 to respiratory tract tissues determine the course of infection. In some COVID-19 patients, an excessive immune response is accompanied by a hyperinflammatory milieu resulting in cytokine storm and acute respiratory distress syndrome (ARDS). These are associated with increased morbidity and mortality, tissue-injuries, and multi-organ failure (Chen et al 2020; Huang et al 2020; Magro 2020; Tang et al 2020; Wang et al 2020; Zhu et al 2020). To evaluate the very first interactions of SARS-CoV-2 patient isolates with human epithelial tissues, 3D models of the human respiratory tract and lung organoids are highly suitable.

By using our established models, we were able to

1. detect that SARS-CoV-2 mediates mucus hypersecretion and mucus plug formation in respiratory tissues, which was illustrated in seriously ill COVID-19 patients with airway obstruction and respiratory failure (Posch et al 2021a; Khan et al 2021);

2. uncover mechanisms of local complement hyperactivation upon SARS-CoV-2 infection of apical-out lung organoids and pseudostratified human airway epithelial cells at an air-liquid interphase. The local complement production aggravated coronavirus infection by triggering release of pro-inflammatory cytokines e.g., IL-1β, IL-6, RANTES, MCP-1 from non-immune epithelial barriers. By blocking C5aR at the basolateral side of the barrier, these effects were reverted, tissue integrity was retained, and virus infection decreased (Posch et al 2021b);

3. identify an antiviral spray (ColdZyme™, Enzymatica) that entirely blocked binding of SARS-CoV-2 and local complement C3 production. This reduced infection and destruction of the tissue model. Our *in vitro* data suggest that ColdZyme™ mouth spray exerts an impact on the prevention of COVID-19. It is important to test the effectiveness of already approved antiviral drugs to check their effectiveness against SARS-CoV-2 (Posch et al 2021c).

These examples, where various 3D tissue model systems provide valuable information on infection processes or novel treatment options, can be expanded further. Human 3D cell culture systems are suitable not merely for studying first host-pathogen interactions and virus dynamics, but they offer added value, especially in the preclinical phase for testing the effects of new therapeutic or repurposed interventions (Djidrovski et al 2021; Raghavan et al 2021).

2.5 Investigation of mitochondrial bioenergetics in 3D models

One of the most frequently applied methods for measuring mitochondrial bioenergetics in 3D models is immunofluorescence. By adding different antibodies to living cells mitochondrial dynamics can be observed in 3D tissue cultures. Not only mitochondria themselves but also the production of proteins involved in mitochondrial metabolism can be examined in 3D models by imaging approaches (Keller et al 2020). The dysfunction of mitochondria contributes to several injuries and diseases. As a new method, the 3D-optical Cryo-Imaging was established to quantify Renal Mitochondrial Bioenergetics Dysfunction in small organs of animals (Mehrvar et al 2021) and is promising for adaption to 3D cell culture models. Additionally, nuclear magnetic resonance spectroscopy was shown to be a powerful method to measure mitochondrial metabolism and respiration in 3D cell models (Hertig et al 2021). Although the measurement of mitochondrial respirometry in a whole 3D culture could be challenging, one can isolate single cells from ALI culture and organoid cultures and perform micro-respirometry analysis (Levitsky et al 2019).

Abbreviations

ALI	air-liquid interphase	IL	interleukin
C3	complement component C3	iPSC	induced pluripotent stem cell
HCS	high content screening	TEER	transepithelial electrical resistance

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