

Available online at www.sciencedirect.com

ScienceDirect

Biomedical Journal

journal homepage: www.elsevier.com/locate/bj

Review Article

3D culture models to study SARS-CoV-2 infectivity and antiviral candidates: From spheroids to bioprinting

Bruna A.G. de Melo ^a, Julia C. Benincasa ^a, Elisa M. Cruz ^a,
Juliana Terzi Maricato ^b, Marimelia A. Porcionatto ^{a,*}

^a Department of Biochemistry, Paulista School of Medicine, Universidade Federal de São Paulo, São Paulo, Brazil

^b Department of Microbiology, Immunology and Parasitology, Paulista School of Medicine, Universidade Federal de São Paulo, São Paulo, Brazil



Dr. Marimelia A. Porcionatto

ARTICLE INFO

Article history:

Received 1 July 2020

Accepted 17 November 2020

Available online 21 November 2020

Keywords:

SARS-CoV-2

COVID-19

Tissue engineering

Organoids

3D bioprinting

ABSTRACT

The pandemic caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is receiving worldwide attention, due to the severity of the disease (COVID-19) that resulted in more than a million global deaths so far. The urgent need for vaccines and antiviral drugs is mobilizing the scientific community to develop strategies for studying the mechanisms of SARS-CoV-2 infection, replication kinetics, pathogenesis, host–virus interaction, and infection inhibition. In this work, we review the strategies of tissue engineering in the fabrication of three-dimensional (3D) models used in virology studies, which presented many advantages over conventional cell cultures, such as complex cytoarchitecture and a more physiological microenvironment. Scaffold-free (spheroids and organoids) and scaffold-based (3D scaffolding and 3D bioprinting) approach allow the biofabrication of more realistic models relevant to the pandemic, to be used as *in vitro* platforms for the development of new vaccines and therapies against COVID-19.

The novel coronavirus disease (COVID-19) emerged at the end of 2019 in the city of Wuhan, China, and by October 2020, it has affected more than 35 million people around the world, according to the World Health Organization (WHO), and the Johns Hopkins Coronavirus Resource Center [1,2]. The viral outbreak is caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which has an increased capacity

of fast spread, most probably due to its affinity with the angiotensin-converting enzyme 2 (ACE2), described as the main receptor for SARS-CoV-2 [3]. As ACE2 is widely distributed in mammalian cells, the virus infects the respiratory tract and rapidly spread throughout the human body infecting other organs, such as the heart, brain, liver, and intestine, as many symptoms related to the multiple-organ infection has

* Corresponding author. Department of Biochemistry, Universidade Federal de Sao Paulo, Sao Paulo 04039-032, Brazil.

E-mail address: marimelia.porcionatto@unifesp.br (M.A. Porcionatto).

Peer review under responsibility of Chang Gung University.

<https://doi.org/10.1016/j.bj.2020.11.009>

2319-4170/© 2020 Chang Gung University. Publishing services by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

been reported clinically [4]. Therefore, due to the severe effects of SARS-CoV-2 in human health, the development of new diagnosis methods, vaccines, and antiviral drugs is urgent.

In vitro models are crucial to validate therapeutics before being used in clinical trials, and can pave the road towards the understanding of SARS-CoV-2 mechanisms of infection and replication. Two-dimensional (2D) cell culture is the standard model in *in vitro* studies, used to comprehend the biology of the virus, as well as for drug screening. Monolayer culture of Vero E6 cells has been an important tool in studies of SARS-CoV-2 infection, such as studies that identified ACE2 as the SARS-CoV receptor and for testing the therapeutic potential of many Food and Administration (FDA) approved drugs [5,6].

Despite its importance, 2D cell culture models fail to recapitulate the complexity of living organisms and often acquire phenotypes that differ significantly from native tissues, which leads to poor prediction of results [7]. Therefore, the use of platforms that provide increased similarity to the *in vivo* physiology and pathology can contribute to advances in the treatments of COVID-19.

In the past years, the three-dimensional (3D) approach has been widely used in cell culture studies, due to their increased capacity of simulating with greater fidelity the cellular microenvironment, as compared to the 2D cell culture, leading to improved cell responses regarding morphology, proliferation capacity, and gene expression profiles [8]. With the advances of tissue engineering, novel technologies have emerged and been used as more realistic *in vitro* models, allowing the construction of complex cytoarchitecture, with better representation of cell heterogeneity, extracellular matrix (ECM) composition, and functionality of native tissues [9]. 3D *in vitro* models consist of scaffold-free (spheroids and organoids) or scaffold-based (3D scaffolding and 3D bioprinting) systems used to study infectivity, replication kinetics, and host–viral interactions of many types of viruses, such as influenza [10,11], syncytial [12], adenovirus [13], norovirus [14], Zika [15], and more recently, SARS-CoV-2 [16], showing increased physiological relevance as compared to 2D models.

Due to the severity and the speed of spread of the pandemic, strategies that contribute to the development of vaccines and drugs, as well as repositioning of currently used drugs, are urgently needed. In this work, we aimed to review the engineered 3D models used as *in vitro* platforms to study infections, host–virus interactions, and drug screening. We believe that these engineered models have great potential to shed light on the mechanisms of SARS-CoV-2 infection and to aid development of vaccines and screen antivirals to treat COVID-19.

The novel coronavirus SARS-CoV-2

Coronaviruses (CoVs) are positive single-stranded RNA viruses from the family Coronaviridae, representing the largest group of viruses that causes respiratory and enteric infections. The direct or indirect interaction between an animal host, such as bats, camels, and other wild animals, and a human being causes the contamination, which spreads rapidly [17]. In

the past 18 years, coronaviruses resulted in three pandemics: Severe Acute Respiratory Syndrome (SARS), caused by SARS-CoV in 2002 [18], Middle East Respiratory Syndrome (MERS), caused by MERS-CoV in 2012 [19] and, currently, a novel Coronavirus Disease, COVID-19, caused by SARS-CoV-2 [20]. Until the present moment, no effective vaccines or treatments have been developed against any human-infecting coronaviruses, although a few broad-spectrum antiviral drugs have shown potential activities [6].

SARS-CoV-2, previously known as 2019-nCoV [21], was first reported in the Chinese city of Wuhan, in December of 2019 [22]. The virus rapidly spread throughout the world leading to an outbreak that is still disrupting health systems, affecting the global economy, and causing severe social impact, due to high hospitalization rates, reduced workforce worldwide, and strict social isolation. The symptoms of the disease range from mild (fever, coughing, sore throat, and fatigue) to severe, such as severe lung injury, pneumonia, bronchiolitis, meningitis, multiple-organ failure, and death [4,23]. In addition to the pulmonary manifestations of COVID-19, which is well described in the literature, it is suggested that other organs can also be infected by SARS-CoV-2, such as the heart, causing myocarditis [24], and the central nervous system, causing headache, anosmia, ageusia, encephalitis and vascular events [25,26].

Although the mechanisms of SARS-CoV-2 infection in the lung and other organs have not been fully elucidated, the literature reports that the virus likely has tropism for different cell types and, therefore, multiple infection routes. The angiotensin-converting enzyme 2 (ACE2) was described as the key receptor for SARS-CoV-2 infections by fusion glycoprotein spike (S) binding and entering the host cell via endocytosis [3,27]. As ACE2 is highly expressed in mammalian organs, it may lead to high invasiveness and multiple tissue damage [28]. It was reported that ACE2 presents a higher affinity with the new coronavirus than with SARS-CoV, which may explain the increased infection rate of SARS-CoV-2 as compared to the previous virus [29]. It is important to highlight the role of the transmembrane serine protease 2 (TMPRSS2), a cellular protease which cleaves SARS-CoV-2 S glycoprotein protein enabling rapid viral internalization and accelerating SARS-CoV-2 replication kinetics in TMPRSS2-expressing cells [30]. More recently, a new route of infection was described by Wang and collaborators, where S protein binds the transmembrane glycoprotein CD147, allowing the virus to enter into the host cell [31]. We emphasize that the work still needs peer review and should be interpreted with caution. Nevertheless, CD147-S route may represent a potential alternative for the development of specific antiviral drugs [32].

Due to the rapid spread and contamination, severity of symptoms, mortality rate, and the unknown long-term effect of SARS-CoV-2 infection, the scientific community joined efforts to understand the mechanisms involved in COVID-19 pathogenicity, transmission, and viral infection of multiple organs, while working on the development of vaccines and new drugs. For these reasons, *in vitro* models using different cell lines became a very useful and powerful tool to accelerate SARS-CoV-2 studies and discoveries. These models are proven to efficiently mimic the physiology of native tissues,

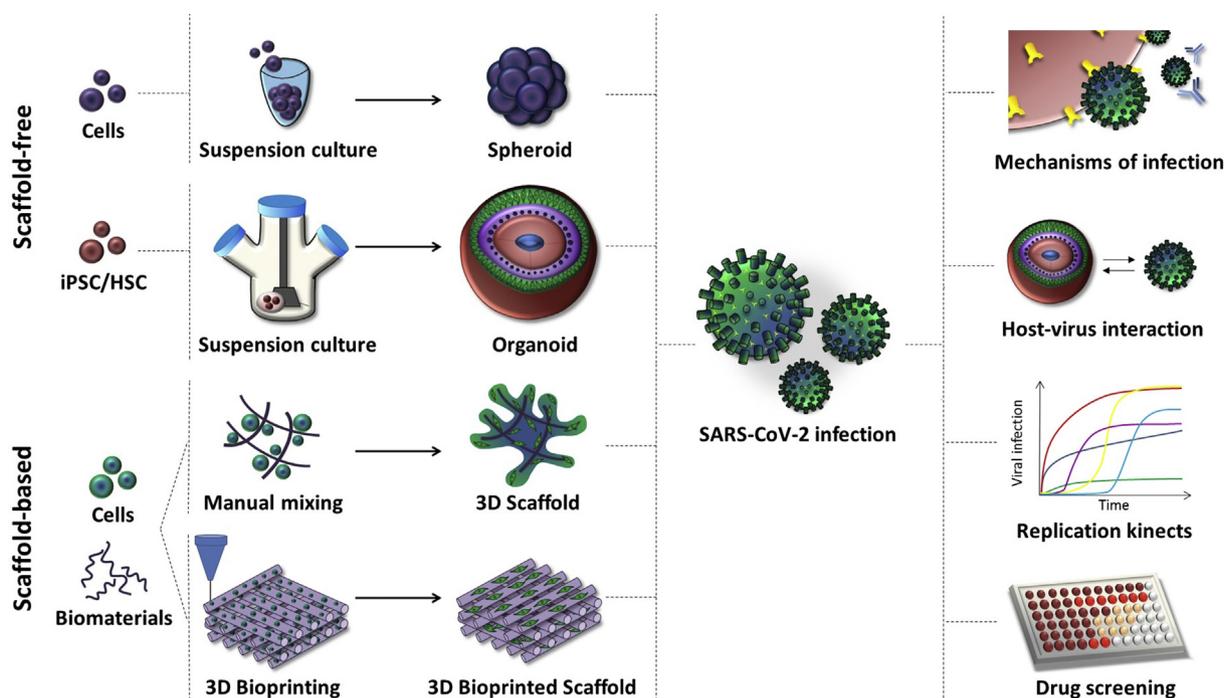


Fig. 1 Tissue engineering strategies to produce 3D *in vitro* models for studying SARS-CoV-2 infection of different organs and tissues, host–virus interaction, replication kinetics and drug screening.

contributing to the studies of several types of diseases, including viral infections in a more physiologic manner [33,34].

2D *in vitro* models used in SARS-CoV-2 studies

Due to the seriousness of the pandemic situation caused by COVID-19, the rapid development of *in vitro* models to study SARS-CoV-2 infection was necessary in order to assist clinical approaches and treatments through the knowledge acquired in basic research, almost in real time. For these purposes, 2D monolayers have been extensively used to study SARS-CoV-2 life cycle and pathogenesis analysis, drug screening and pre-clinical evaluation of antiviral potential, and cytopathic effect of candidate molecules [35].

Vero cells E6 cells, isolated from African green monkeys kidneys, are susceptible to many types of viruses, including the SARS-CoV [36] and SARS-CoV-2 [37]. They produce high viral titers, probably due to the expressive presence of ACE-2 in their apical region, and because these cells do not produce type I interferons (IFN) when infected by several viruses. This phenomenon is due to a deletion of ~9 Mbp deletion on chromosome 12, which when in homozygosis, results in a more permissive phenotype for viruses. Thus, the IFN deficiency allows SARS-CoV-2 to replicate sustainably in Vero cells [38]. This cell line was used in some important studies involving SARS-Cov-2, such as for identifying the ACE2 as the functional receptor of SARS-CoV, for demonstrating that anti-ACE2 acted as an inhibitor of viral replication in these cells [5], for identifying other potential routes of infection [31], and for testing the inhibition potential of antiviral candidates [6].

However, the highly permissive phenotype Vero cells have some limitations, as it does not accurately represent the pathogenesis of COVID-19, as its initial target organs are the air and pulmonary epithelia and the venous endothelium. Therefore, other cell types seem to serve as *in vitro* models that may better recapitulate the real physiology of the disease.

Primary Human Airway Epithelial Cells (HAE) are now commercially available and have been of great utility in studies involving coronaviruses. Besides being efficient for SARS-CoV-2 (and other similar viruses) isolation, these cells mimic infected human lung cells, and it is clearly possible to observe cytopathic effects about 96 h after SARS-CoV-2 infection. However, an important disadvantage is that these cells have limited replication capacity, requiring constantly acquisition of new stocks [20,39]. Besides HAE, it was reported that primary human nasal epithelial, large airway epithelial (bronchi and large airway epithelial), lower airway epithelial (bronchiolar and small airway epithelial) [40], type I and type II pneumocytes (AT1 and AT2) [41], and primary enterocytes [42,43] supports SARS-CoV-2 infection and replication, as well as primary neurons and neural stem cells (NSCs) [44,45]. However, obtaining all these cell types is difficult and limited, besides the high costs.

To circumvent this problem, many studies have used the most diverse types of proliferating cells lineages, usually derived from tissue-specific carcinomas. Among them, we can highlight other cell lines recently used in studies involving SARS-CoV-2, such as the pulmonary cell lines BEAS-2B (human bronchial epithelium) and A549 (adenocarcinomic human alveolar basal epithelial cells). BEAS-2B, appears to have an accelerated viral replication kinetics, in addition to produce higher viral loads than A549, probably due to its

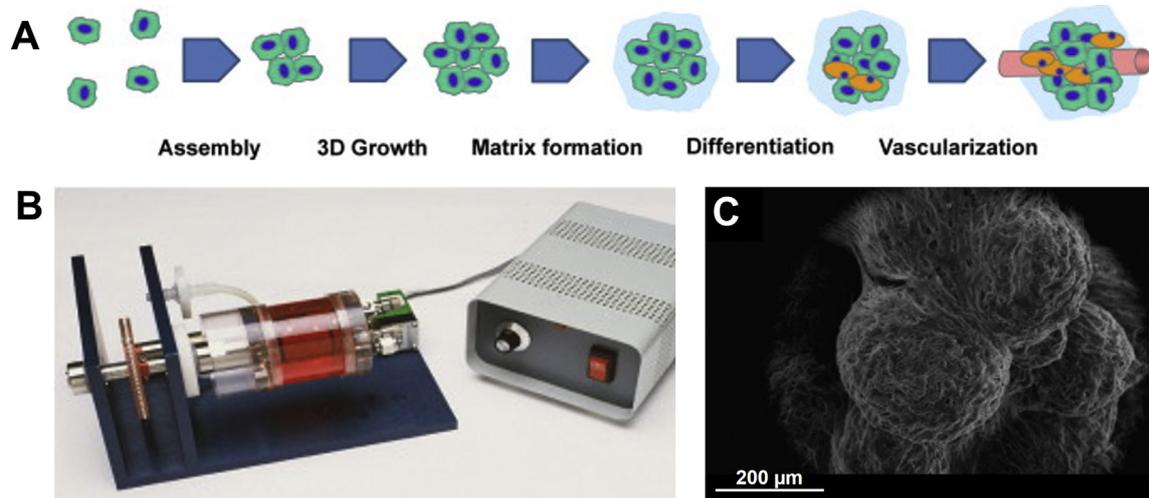


Fig. 2 Fabrication of spheroids using a rotating wall vessel bioreactor. (A) Scheme illustrating the spheroids assembly and formation in the rotating wall vessel bioreactor. (B) Image of the bioreactor system. (C) Scanning electron microscopy of human neural progenitor spheroids used in SARS-CoV infection studies. Reproduced with permission from Elsevier [60].

higher ACE-2 and TMPRSS2 expression levels [30]. Calu-3 cells, another human pulmonary cell lineage, isolated from non-small cell lung cancer, has shown to have great permissiveness and increased viral load when infected with SARS-CoV-2, becoming a promising cell line to be used in COVID-19 *in vitro* studies [46]. Other cell lineages, mainly Caco-2 cells (human colon adenocarcinoma) and HEK293T (human embryonic kidney (HEK) grown in tissue culture) are rising as alternative models for SARS-CoV-2 *in vitro* infection to study the viral tropism of human non-pulmonary cells [47,48]. However, both cell lines present low levels of SARS-CoV-2 replication in culture.

Although these cell lineages are mainly used in 2D cell culture approach, they are promising elements for the bio-fabrication of *in vitro* 3D models, which can better represent the hosts' physiological environment where SARS-CoV-2 infection naturally occurs.

2D vs 3D *in vitro* models in virology

Conventional 2D cell cultures have greatly contributed to the understanding of host cell–virus interactions, mechanisms of virus transmission, replication, and adaptation, as well as screening of antiviral drugs [6,37]. However, this model have some limitations that rely on the difficulty of reconstituting the accurate and complex microenvironment found in living organisms. Cell–cell junctions, apical-basal polarity, and cell communication through gradients of endogenous growth factors, chemokines, and nutrients may be inadequate to guarantee the similarity with an *in vivo* system [7]. These limitations exemplify the necessity to develop new platforms for *in vitro* modeling [7,49]. A 3D cell culture approach represents a more realistic environment for cells, contributing to the cell adhesion, maintenance of cytoarchitecture, perception of the mechanical stimulus, and cell signaling, which in turn, regulate functional responses that differ from traditional 2D cultures [50].

Many works in the literature reported comparative studies of monolayer cells and 3D cultures infected with different types of viruses, such as poxviruses [51,52], adenovirus [53], hepatitis B and C [53,54], Zika [15,55] and influenza [10]. These works showed that different approaches of 3D cell culture, such as spheroids, organoids, 3D scaffolds and bioprinted structures were able to mimic many types of tissues using different cell lines, presenting higher sensitivity in virus isolation and specificity of several antiviral compounds than conventional 2D models. Therefore, scaffold-free or scaffold-based 3D cell culture may serve as better *in vitro* models to study SARS-CoV-2 infection mechanisms, replication, pathophysiology of injured organs and tissues, and to provide high-throughput drug screening strategies [Fig. 1].

SARS-CoV-2 infection in 3D scaffold-free models

Spheroids

Spheroids are cellular aggregates that self-assemble when cultured on non-adhesive surfaces, preserving cell–cell interactions and tissue-specific phenotype [33]. This cell culture method is widely used in studies of virus infection [12,13,55–57], being fabricated by culturing cells in non-adherent cell culture flasks and wells [58], non-adherent spheroid molds [59], bioreactors [60] or by the hanging drop method [53].

Rosellini et al. reported the fabrication of spheroids using Vero E6 and A549 cells, among other cell lines, aiming to study the sensitivity and isolation of cytomegalovirus, adenovirus, and herpes simplex virus [53]. Results showed that 3D spheroids, infected by the three viruses, were more sensitive to infection than 2D monolayers of the same cell lines, in addition to express viral proteins faster, probably due to the increased number of viral receptors in the spheroids. Recently, Saleh et al. fabricated A549 spheroids by seeding the cells in an ultra-low attachment 96-well plate to mimic the

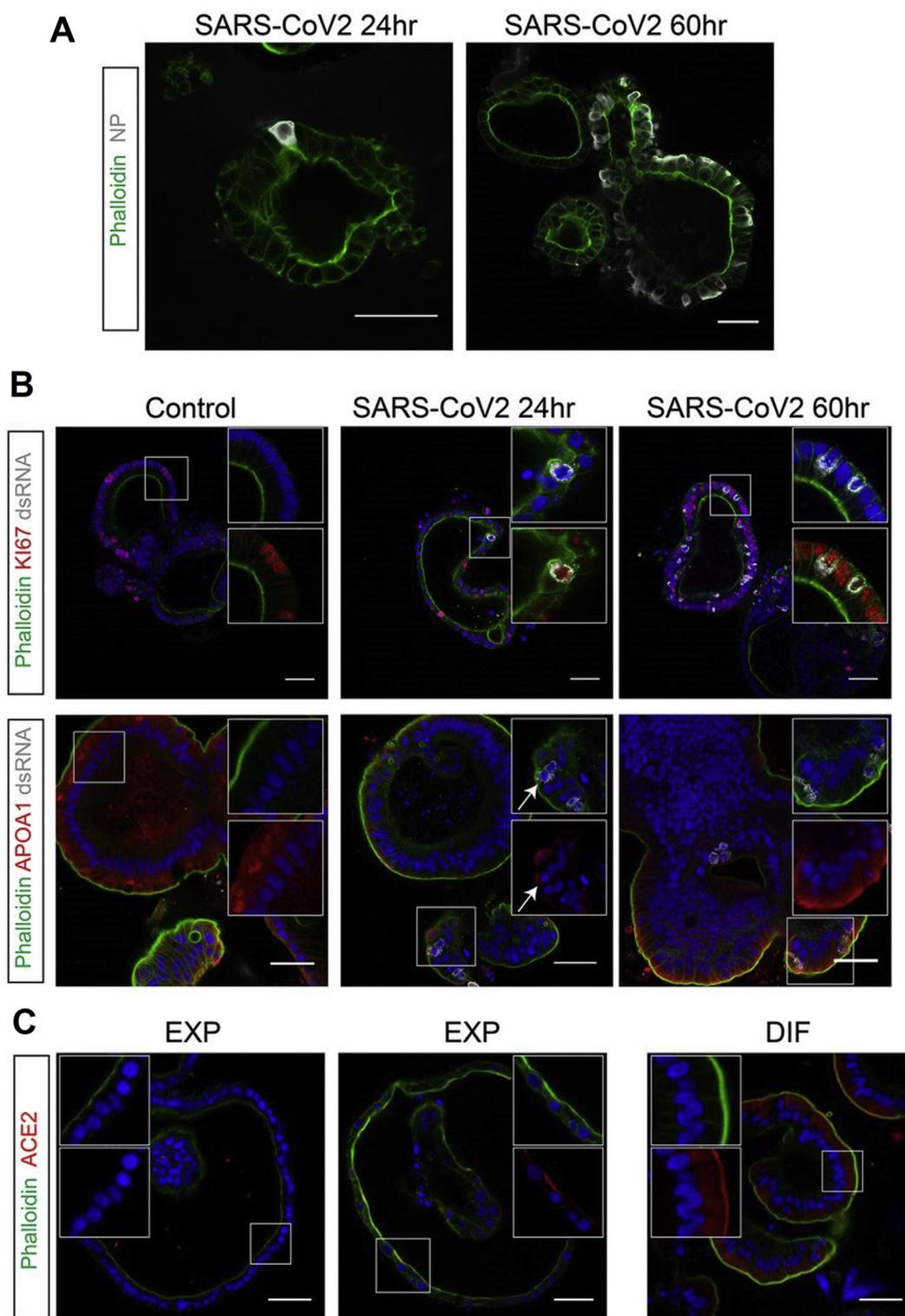


Fig. 3 SARS-CoV-2 infecting intestinal organoids. (A) Immunofluorescent images showing the progressive SARS-CoV-2 infection, being possible to observe the infection clusters spreading through the organoid after 60 days. NP = Nucleoprotein. (B) Immunofluorescent images of dividing cells (KI67-positive) and post-mitotic enterocytes identified by Apolipoprotein A1 (APOA1) (pointed arrows). (C) ACE2 staining in intestinal organoids in expansion (EXP) and differentiated (DIF). Reproduced with permission from AAAS [42].

alveolar tissue and study respiratory syncytial virus pathogenesis [12]. Results showed that the 3D model was permissive to the virus, leading to syncytia spread towards the nucleus and fast cell death at the core of the spheroids, suggesting the model is promising for studies of infections *in vitro*.

SARS-CoV infection in lung and brain *in vitro* models was evaluated using spheroids composed of human bronchial tracheal cells/BEAS-2B cells and human neural progenitor cells, respectively, formed in a rotating wall vessel bioreactor [Fig. 2] [60]. Both spheroids types showed cytopathic effects in response to SARS-CoV infection, suggesting a similar behavior to the native system, being promising for estimating the pathophysiology of the lung, brain, and other tissues infected with SARS-CoV-2.

Neurospheres, fabricated using neural stem cells cultured in a non-adherent surface, were used to study molecular mechanisms implicated in brain malformation due to Zika virus infection [15,61]. In these studies, it was observed that the viral infection impaired growth and disrupted morphogenesis of infected neurospheres by inducing cell death, indicating that the neurospheres represent a suitable model to study the mechanisms and effects of Zika infection. In another approach, Bullen et al. biofabricate induced pluripotent stem cells (iPSCs)-derived BrainSpheres to study neurotropism of SARS-CoV-2 [62]. Viral infection and replication was observed in the 3D model, probably due to the high expression of the ACE2 receptor gene. However, TMPRSS2 was not expressed, suggesting an alternative route.

Although spheroids have shown an increased capacity to respond to virus infection over 2D monolayers, they still lack the biological complexity that may be found in other 3D models that possess increased complexity, such as organoids.

Organoids

Organoids are self-organizing structures established from organ-specific cell types [iPSCs or multipotent adult tissue stem cells (ASCs)] that retain multicellular diversity, cytoarchitecture at early development, and functional hallmarks to their counterpart organs and tissues *in vivo* [63]. These complex 3D culture systems have been used to mimic multiple types of tissues, such as brain, lung, intestine, and liver to study host–virus interactions for important viruses, such as the Zika [15,64], influenza viruses [11], noroviruses [14], hepatitis C [65], and MERS-CoV [66]. In all cases, results showed that the organoids simulated the native tissues morphologically and functionally, indicating they are suitable to be used as *in vitro* models to study infectivity.

Since the COVID-19 outbreak, many studies involving SARS-CoV-2-host interactions using organoids have been reported, with results indicating their potential to contribute to the development of treatments and drug candidates [67].

The respiratory tract is the first target for SARS-CoV-2, and respiratory symptoms correspond to the main clinical presentation of COVID-19. In a preliminary study, human bronchial organoids fabricated using commercial human bronchial epithelial cells presented high expression of ACE2 and were successfully infected by SARS-CoV-2, which was also replicated in this model [68]. In addition, camostat, a therapeutic candidate against COVID-19 acting through transmembrane

serine protease 2 (TMPRSS2) inhibition, was tested and results showed that, after the treatment of infected organoids, SARS-CoV-2 viral genome reduced to 2%. Lung organoids were also used to high throughput screen the FDA-approved drugs imatinib, mycophenolic acid, quinacrine dihydrochloride, and chloroquine, and evaluate their capacity to inhibit SARS-CoV-2 entry [69]. Initially, it was verified the permissiveness of lung cell to the virus entry, due to their expression of ACE2. Then, organoids were treated with each drug following exposition to the virus, with results showing a blockage of luciferase activity in a dose-dependent manner after treatment. These results indicate that organoids may recapitulate the native tissues morphology, physiology, and functionality, with great potential to contribute to studies of infection mechanisms, immunological responses to the virus, and screening of therapeutics.

Even though respiratory symptoms are the clinical predominance of COVID-19 patients, a subset of manifestations may appear in different organs due to the ubiquitous distribution of ACE2 throughout the organism, as mentioned before. Because SARS-CoV-2 was identified in the urine of patients [70], it can be assumed that the virus spreads from the lungs to the kidney through the vasculature. Therefore, Monteil et al. used iPSCs to produce human capillary and kidney organoids for evaluating the infectivity of SARS-CoV-2, as well as the inhibitory effect of human recombinant soluble ACE2 (hrsACE2) [37], which has been considered a potential novel treatment for COVID-19. Results showed that both vascular and kidney organoids were infected with SARS-CoV-2, and the supernatant was also capable to infect monolayers of Vero E6 cells. Besides, SARS-CoV-2 entry into the organoids was significantly reduced by hrsACE2 in the early stages of infection.

ACE2 is also highly expressed in multiple digestive tract organs, which can lead to infection by SARS-CoV-2 and consequently gastrointestinal symptoms, being observed in many patients [71]. Lamers et al. fabricated human small intestinal organoids from primary gut epithelial stem cells and evaluated SARS-CoV and SARS-CoV-2 infection capacity [42]. Results showed that both viruses were capable to infect enterocyte lineage cells in the organoids [Fig. 3]. In another study, Zhang et al. reported the biofabrication of human intestinal enteroids to study SARS-CoV-2 infection [43]. It was observed the capacity of the organoids to be infected by the virus, which replication was observed in human duodenum, ileum and colon organoids, with virus levels increasing about 1000-fold after 24 h of infection. Bat and human intestinal organoids were fabricated aiming to obtain *in vitro* models of the potential origin of SARS-CoV-2 and of a human organ that is a potential transmission route, respectively [16]. Bat intestinal organoids corroborated the multicellular composition present in the native epithelium and were able to replicate the SARS-CoV-2, indicating its capacity to support natural infection. Human epithelial organoids were also capable of replicating SARS-CoV-2 and inducing type III IFNs and inflammatory mediators, which are upregulated upon viral infections in the human intestine. These human organoids can be used as a powerful instrument to evaluate the intestinal tract as a potential transmission route of SARS-CoV-2.

Liver abnormalities have become a common hallmark in COVID-19 patients [72,73]. Recently, it was reported that

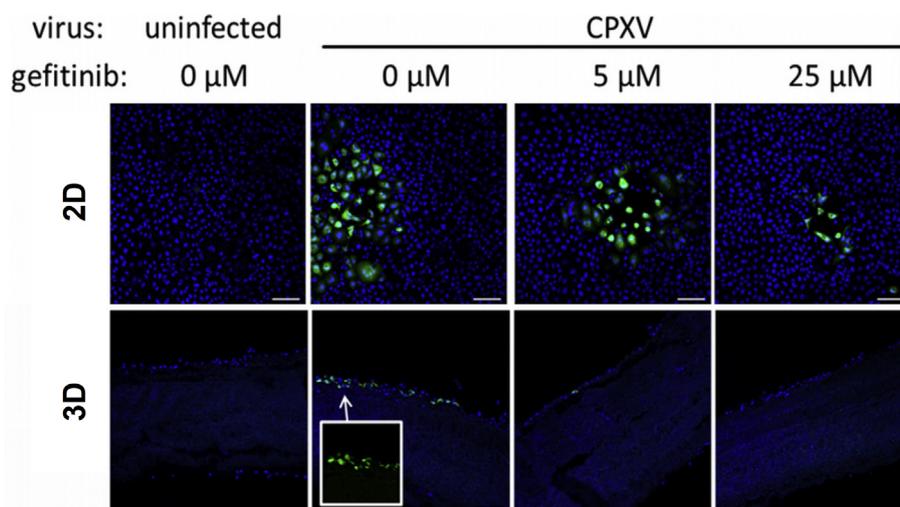


Fig. 4 Comparison between 2D and 3D models after infection with Cowpox virus (CPXV) for 48 h and treated with different concentrations of gefitinib. Immunofluorescence images showed that gefitinib strongly inhibited infection in 3D model when treated with the lowest concentration of the inhibitor, while cells in the 2D model remained infected even at 25 μM of gefitinib. Infected cells were visualized with GFP (green) and cellular nuclei was visualized with DAPI (blue). Scale bar = 100 μm . Reproduced with permission from Elsevier [52].

cholangiocytes, epithelial cells of the bile ducts that express both ACE2 and TMPRSS2, are subject to the SARS-CoV-2 infection [30,74]. To analyze the mechanisms of virus infection and direct biliary cell injury, Zhao et al. produced the first liver ductal organoids, using cholangiocytes expressing human-specific ACE2+/TMPRSS2+ [75]. The proposed model was permissive to SARS-CoV-2 infection and supported viral replication. Molecular studies revealed a gene expression modulation that lead to a disruption in bile acid transporting functions, impairing liver functions.

Brain organoids have also been biofabricate using human iPSCs to study brain cells behavior in the presence of SARS-CoV-2. For instance, Jacob et al. studied SARS-CoV-2 neurotropism using human iPSC-derived brain organoids as an *in vitro* infection platform [76]. In this study, organoids of specific brain regions, such as cerebral cortex, hippocampus, hypothalamus, and midbrain were exposed to SARS-CoV-2 for 8 h. Results showed an increased number of neurons infected in all organoids, as compared to other neural cell types, and a stabilization of the number of infected cells, indicating that the virus may not spread among neurons. Another preliminary study reported the fabrication of human iPSCs-derived brain organoids to study the neurodegenerative effects of SARS-CoV-2 on the central nervous system [77]. In this work, the virus also preferably targeted human cortical neurons instead of neural stem cells within the brain organoids, demonstrating that developing embryonic brains are less vulnerable. Similarly to that observed in the work previously discussed, the virus could not replicate, the opposite of what was observed for kidney and intestinal organoids, suggesting that the central nervous system may not support SARS-CoV-2 replication. More recently, Pedrosa et al. reported similar results, in which neurospheres, mainly composed of NSCs, showed not to be permissive to SARS-CoV-2 infection [44]. Controversially, Song et al. reported a detailed study of SARS-CoV-2 infection of mature neuronal fibers within brain

organoids, as early as 24 h post-infection. Moreover, they demonstrated that the *in vitro* infection was able to induce metabolic changes in these cells [45].

As aerosol transmission is the main mechanism of spreading the virus, Makovoz et al. aimed to study the tropism of SARS-CoV-2 for ocular cell types in eye organoids, as a potential entry route [78]. The organoid produced from human pluripotent stem cell enabled the researchers to observe SARS-CoV-2 RNA present in several eye regions, such as cornea, sclera, limbus iris, retinal pigment epithelium, and choroid, with low replication in the central cornea. In addition, due to the high expression of ACE2 and TMPRSS2, ocular surface ectoderm was also permissive to virus infection, suggesting the risk of contamination by SARS-CoV-2 regardless wearing facemasks.

Some of the challenges in culturing organoids are related to the lack of vascularization, neuronal circuit, immune system, reproducibility, and preclinical validation [79–81]. However, the results discussed above indicate that organoids not only are capable of recapitulate the native tissue morphology, physiology, and functionality, but also may substantially contribute to studies of SARS-CoV-2 infection mechanisms, drug screening and disease research.

SARS-CoV-2 infection in 3D scaffold-based models

Conventional 3D scaffolding

In tissue engineering, 3D scaffold-based models present some advantages over spheroids and organoids, especially due to the presence of biomaterials that simulate the ECM microenvironment. This system can be fabricated using many types of biocompatible materials, naturally present in the ECM or not, providing mechanical strength, physical stability, and

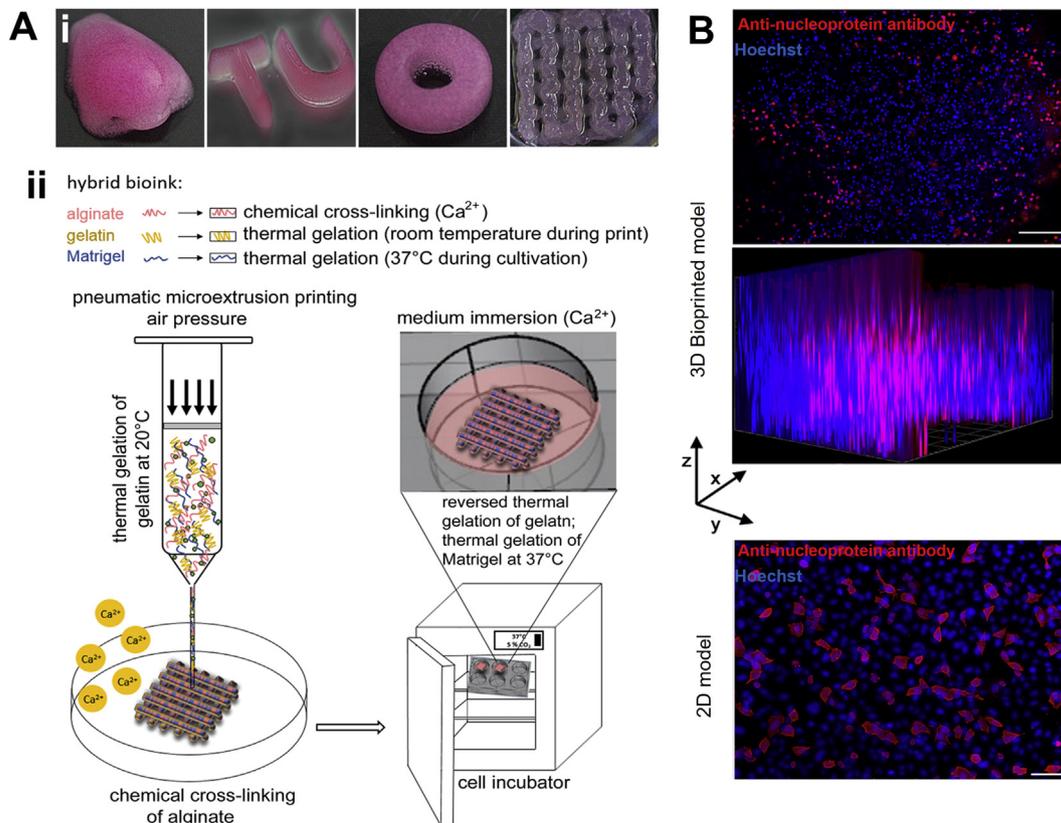


Fig. 5 3D bioprinting lung model for influenza A infection. (A) 3D bioprinted structures with different shapes (i) and the schematic illustration of the bioprinting process, in which alginate, gelatin, and Matrigel™ are extruded, followed by the alginate crosslinking with calcium ions (Ca^{2+}), and the removal of gelatin during incubation at 37°C . (B) Immunohistochemistry of the 3D bioprinted construct and 2D monolayer infected with H3N2 strain for 24 h, and stained with anti-nucleoprotein antibody, showing the widespread distribution of the virus in the bioprinted model. Reproduced from Nature Research [93]. <http://creativecommons.org/licenses/by/4.0/>.

biological features that stimulate cell behavior, such as migration, proliferation, and differentiation [82]. In SARS-CoV-2 infection studies, the presence of an ECM may have important effects on cellular responses, as coronaviruses bind to ECM components, such as heparan sulfate, to assist its infection in the host [83–85].

Aiming the fabrication of a more realistic model of lung tissue to study influenza A infections, Bhowmick et al. used airway epithelial cells mixed to a collagen-chitosan polymeric blend, simulating the alveolar barrier structure [10]. Cells cultured in the 3D matrix presented increased resemblance to the native tissue concerning cell morphology, specific markers expression, and differentiation, as compared to a 2D culture. In addition, the 3D model showed immune responses that better resembled that of the native system when infected with H1N1 and H3N2 strains. The mechanisms of hepatitis B infection in the liver was evaluated by Zhang et al. which used a 3D model fabricated with the native ECM to support human hepatocytes [54]. The liver ECM was decellularized, maintaining its biological properties, which contributed to the construction of a more realistic *in vitro* model. Results showed that, compared to the monolayer cell culture, the 3D model significantly increased the infection level, efficiency, as well as HBsAg secretion. Besides, it was an efficient model to test

infection inhibition, which was made using the anti-hepatitis B entecavir that was introduced into the system, leading to a suppression of the virus DNA. Koban et al. also used decellularized ECM mixed to human keratinocytes to study the antiviral activity of gefitinib against the Cowpox virus [52]. The 3D cell culture showed higher sensitivity to the infection, as compared to a 2D monolayer, in addition to showing that virus replication was significantly low in the presence of gefitinib [Fig. 4]. The authors suggested that the 3D model could be easily adapted to other cell types, being promising to study the antiviral activity against other types of virus, and therefore, could be a potential alternative to test drugs against SARS-CoV-2.

3D bioprinted models

The 3D bioprinting is an emerging technology that has been widely employed in the tissue engineering field aiming to optimize the conventional 3D cell culture. Due to its capacity to deposit layer-by-layer cells and biomaterials in an organized and automatized manner, through a computer-aided process, the fabrication of complex architectures that mimic the structure of organs and tissues became possible [86,87]. Several bioprinting methods have been employed to construct

mimetic tissues, each presenting advantages and limitations. For instance, extrusion-based bioprinting, inkjet/drop-on-demand, laser-assisted, stereolithography, and electrospinning-based are some methods used in biofabrication, being the extrusion-based bioprinting technique the most commonly used, due to its easy of handling and low cost [88,89].

Therefore, the construction of more realistic models in terms of cell heterogeneity, ECM presence, and the complex organization has greatly improved the physiological and pathological cell microenvironment, contributing significantly to cell response and prediction of results of *in vitro* studies [90], including those involving viral infections [91–93].

To study the mechanisms of SARS-CoV-2 infection in the lung, the alveolar tissue can be mimicked using a 3D bioprinted airway epithelium [94,95]. Horvath et al. reported the 3D bioprinting of an alveolar barrier by using a micro-extrusion bioprinter [94]. The biofabricated structure was morphologically similar to the native tissue, being highly organized in a thin layer. On the other hand, cells manually mixed to Matrigel™ formed multi-layered clusters with tick ECM between the epithelial and endothelial cells, which can affect the permeability of biomolecules. This work showed that 3D bioprinting technology offers relevant advantages over conventional 3D scaffolding, with potential to be used for testing drug candidates that inhibit SARS-CoV-2 infection and replication. The effects of influenza A infection was modeled using an engineered respiratory tract [93]. For this, human alveolar epithelial (A549) cells were embedded in a polymeric blend composed of gelatin (to assure printability), alginate (for structural stability), and Matrigel™ (to assure biocompatibility), being extruded using micro-extrusion bioprinting technology [Fig. 5A]. Results showed that the virus was homogeneously distributed throughout the bioprinted construct, which reflects the natural condition of infection [Fig. 5B]. The virus was capable to replicate in the 3D model, and the bioprinted cells also showed *in vivo*-like immune response, observed by the inflammatory cytokine interleukin 29 release. Hiller et al. used human liver cells (HepaRG) embedded in gelatin/alginate/decellularized ECM as bioink to bioprint a liver tissue-like structure using an extrusion-based bioprinter [92]. The 3D model was used to test adenovirus infection, which showed high capacity to support a widespread infection and virus replication.

Although there is still a void in the literature regarding 3D bioprinting to study viral infections, the technology has shown great potential in constructing refined biological models with high fidelity regarding the native tissues. These models would greatly contribute to the understanding of the mechanisms of infection and replication kinetics of SARS-CoV-2 in human organs, in addition to provide *in vitro* platforms to test vaccines and drugs to treat COVID-19.

Conclusion and future perspectives

As the COVID-19 outbreak continues to affect thousands of people around the world, it is urgent the development of strategies that lead to effective treatments and vaccines.

Although 2D conventional cell culture has shown to be an important tool in virology studies, this model fails in replicating the cellular microenvironment in terms of architecture, composition, physiological function, and mechanical stimulus, which may lead to low prediction of results [7]. The establishment of 3D cell culture and the biofabrication of tissue-like structures can mimic the complex microenvironment found in the many organs affected by SARS-CoV-2 with higher accuracy, providing robust data to elucidate cellular and molecular mechanisms of virus infection, replication kinetics, and host–virus interaction.

In this work, we reviewed the strategies offered by the tissue engineering field to biofabricate useful *in vitro* 3D models to understand the effects of SARS-CoV-2 infection. Spheroids have been an important tool to study the effects of Zika virus in neurogenesis during brain development [15,55], and have shown cytopathic effects in lung and brain spheroids in response to SARS-CoV infection [60]. Given the numerous evidences of neurological disorders in COVID-19, neurospheres can elucidate the mechanisms of neural injury. Although spheroids lack in biological function and structure complexity, they can be fabricated by using simple methodologies. Organoids, in contrast, are more complex and organized in terms of structure and composition, resembling the natural environment of the target organ [79]. Due to their cytoarchitecture, organoids have been widely used to construct mimetic organs to study SARS-CoV-2 and its subsequent effects after infection. Although this model face some limitations [81,96], similarities to native tissues morphology, physiology, and functionality are leading to reliable results, which can accelerate the process of developing new drugs and consequent control of the disease.

Regarding scaffold-based strategies, these 3D models have been used to study the infectivity of different types of viruses, indicating they can greatly contribute to the understanding of the mechanisms of SARS-CoV-2 infection. By using 3D bioprinting technology, it is possible to fabricate complex architectures that resembles native tissues in a reproducible manner, providing molecular and cellular machinery to effectively replicate SARS-CoV-2, holding promises to future drug tests [94].

Based on results of previous studies, *in vitro* 3D models are attractive alternatives to be used in virology, and this review hopes to shed light on different strategies to study the effects of SARS-CoV-2 in human organs. We believe that the 3D cell culture approach can greatly contribute to the development of faster diagnostics and therapeutics, due to its capacity to mimic with greater fidelity the *in vivo* system, providing increased prediction of results and faster translational applications to treat patients affected by SARS-CoV-2.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by The São Paulo Research Foundation (FAPESP), grant numbers 2018/23039-3, 2018/12605-8, and 2020/05289-2; National Council for Scientific and Technological Development, grant numbers 465656/2014-5, 309679/2018-4; and Coordination for the Improvement of Higher Education Personnel, financial code 001.

REFERENCES

- [1] World Health Organization. Coronavirus disease (COVID-19) pandemic 2020. <https://www.who.int/emergencies/diseases/novel-coronavirus-2019>. [accessed 6 October 2020].
- [2] Johns Hopkins University & Medicine. Coronavirus Resource center. 2020. <https://coronavirus.jhu.edu/>. [accessed 6 October 2020].
- [3] Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell* 2020;181:281–292.e6.
- [4] Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* 2020;395:507–13.
- [5] Li W, Moore MJ, Vasilieva N, Sui J. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 2003;426:450–4.
- [6] Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res* 2020;30:269–71.
- [7] Duval K, Grover H, Han LH, Mou Y, Pegoraro AF, Fredberg J, et al. Modeling physiological events in 2D vs. 3D cell culture. *Physiology* 2017;32:266–77.
- [8] Khademhosseini A, Langer R. A decade of progress in tissue engineering. *Nat Protoc* 2016;11:6–9.
- [9] Huh D, Hamilton GA, Ingber DE. From 3D cell culture to organs-on-chips. *Trends Cell Biol* 2011;21:745–54.
- [10] Bhowmick R, Derakhshan T, Liang Y, Ritchey J, Liu L, Gappa-Fahlenkamp H. A three-dimensional human tissue-engineered lung model to study influenza A infection. *Tissue Eng* 2018;24:1468–80.
- [11] Zhou J, Li C, Sachs N, Chiu MC, Wong BHY, Chu H, et al. Differentiated human airway organoids to assess infectivity of emerging influenza virus. *Proc Natl Acad Sci U S A* 2018;115:6822–7.
- [12] Saleh F, Harb A, Soudani N, Zaraket H. A three-dimensional A549 cell culture model to study respiratory syncytial virus infections. *J Infect Public Health* 2020;13:1142–7.
- [13] Grill J, Lamfers MLM, van Beusechem VW, Dirven CM, Pherai DS, Kater M, et al. The organotypic multicellular spheroid is a relevant three-dimensional model to study adenovirus replication and penetration in human tumors in vitro. *Mol Ther* 2002;6:609–14.
- [14] Ettayebi K, Crawford SE, Murakami K, Broughman JR, Karandikar U, Tenge VR, et al. Replication of human noroviruses in stem cell-derived human enteroids. *Science* 2016;353:1387–94.
- [15] Cugola FR, Fernandes IR, Russo FB, Freitas BC, Dias JLM, Guimarães KP, et al. The Brazilian Zika virus strain causes birth defects in experimental models. *Nature* 2016;534:267–71.
- [16] Zhou J, Li C, Liu X, Chiu MC, Zhao X, Wang D, et al. Infection of bat and human intestinal organoids by SARS-CoV-2. *Nat Med* 2020;26:1077–83.
- [17] Yang W, Sirajuddin A, Zhang X, Liu G, Teng Z, Zhao S, et al. The role of imaging in 2019 novel coronavirus pneumonia (COVID-19). *Eur Radiol* 2020;30:4874–82.
- [18] Drosten C, Günther S, Preiser W, Van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* 2003;348:1967–76.
- [19] de Groot RJ, Baker SC, Baric RS, Brown CS, Drosten C, Enjuanes L, et al. Middle East respiratory syndrome coronavirus (MERS-CoV): announcement of the coronavirus study group. *J Virol* 2013;87:7790–2.
- [20] Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med* 2020;382:727–33.
- [21] Gorbalenya AE, Baker SC, Baric RS, de Groot RJ, Drosten C, Gulyaeva AA, et al. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat Microbiol* 2020;5:536–44.
- [22] Bogoch II, Watts A, Thomas-Bachli A, Huber C, Kraemer MUG, Khan K. Pneumonia of unknown aetiology in Wuhan, China: potential for international spread via commercial air travel. *J Trav Med* 2020;27:taaa008.
- [23] Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. A new coronavirus associated with human respiratory disease in China. *Nature* 2020;579:265–9.
- [24] Li JW, Han TW, Woodward M, Anderson CS, Zhou H, Chen YD, et al. The impact of 2019 novel coronavirus on heart injury: a Systematic review and Meta-analysis. *Prog Cardiovasc Dis* 2020;63:518–24.
- [25] Zubair AS, McAlpine Lindsay S, Gardin T, Farhadian S, Kuruvilla DE, Spudich S. Neuropathogenesis and neurologic manifestations of the coronaviruses in the age of coronavirus disease 2019. *JAMA Neurol* 2020;77:1018–27.
- [26] Wu Y, Xu X, Chen Z, Duan J, Hashimoto K, Yang L. Nervous system involvement after infection with COVID-19 and other coronaviruses. *Brain Behav Immun* 2020 July;87:18–22.
- [27] Tortorici MA, Veesler D. Structural insights into coronavirus entry. *Adv Virus Res* 2019;105:93–116.
- [28] Devaux CA, Rolain JM, Raoult D. ACE2 receptor polymorphism: susceptibility to SARS-CoV-2, hypertension, multi-organ failure, and COVID-19 disease outcome. *J Microbiol Immunol Infect* 2020;53:425–35.
- [29] Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* 2020;367:1260–3.
- [30] Hoffmann M, Kleine-weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 2020;181:271–80.
- [31] Wang K, Chen W, Zhou YS, Lian JQ, Zhang Z, Du P, et al. SARS-CoV-2 invades host cells via a novel route: CD147-spike protein. *BioRxiv* 2020. 03.14.988345 [Preprint]. Available from: <http://biorxiv.org/content/early/2020/03/14/2020.03.14.988345>.
- [32] Ulrich H, Pillat MM. CD147 as a target for COVID-19 treatment: suggested effects of azithromycin and stem cell engagement. *Stem Cell Rev Rep* 2020;16:434–40.
- [33] He B, Chen G, Zeng Y. Three-dimensional cell culture models for investigating human viruses. *Virology* 2016;31:363–79.
- [34] Gardner JK, Herbst-kralovetz MM. Three-dimensional rotating wall vessel-derived cell culture models for studying virus-host interactions. *Viruses* 2016;8:304.
- [35] Takayama K. In vitro and animal models for SARS-CoV-2 research. *Trends Pharmacol Sci* 2020;41:513–7.

- [36] Ng ML, Tan SH, See EE, Ooi EE, Ling AE. Early events of SARS coronavirus infection in Vero cells. *J Med Virol* 2003;331:323–31.
- [37] Monteil V, Kwon H, Prado P, Hagelkrüys A, Wimmer RA, Stahl M, et al. Inhibition of SARS-CoV-2 infections in engineered human tissues using clinical-grade soluble human ACE2. *Cell* 14 May 2020;181(4):905–13.
- [38] Osada N, Kohara A, Yamaji T, Hirayama N, Kasai F, Sekizuka T, et al. The genome landscape of the african green monkey kidney-derived Vero cell line. *DNA Res* 2014;21:673–83.
- [39] Jonsdottir HR, Dijkman R. Coronaviruses and the human airway: a universal system for virus-host interaction studies. *Virol J* 2016;13:24.
- [40] Zhu N, Wang W, Liu Z, Liang C, Wang W, Ye F, et al. Morphogenesis and cytopathic effect of SARS-CoV-2 infection in human airway epithelial cells. *Nat Commun* 2020;11:1–8.
- [41] Ling T, Kuo M, Li C, Yu AL, Huang Y, Wu T, et al. Identification of pulmonary Oct-4+ stem/progenitor cells and demonstration of their susceptibility to SARS coronavirus (SARS-CoV) infection in vitro. *Proc Natl Acad Sci Unit States Am* 2006;103:9530–5.
- [42] Lamers MM, Beumer J, J van der Vaart, Knoop K, Puschhof J, Breugem TI, et al. SARS-CoV-2 productively infects human gut enterocytes. *Science* 2020;1669:1–11.
- [43] Zang R, Castro MFG, McCune BT, Zeng Q, Rothlauf PW, Sonnek NM, et al. TMPRSS2 and TMPRSS4 promote SARS-CoV-2 infection of human small intestinal enterocytes. *Sci Immunol* 2020;5:1–15.
- [44] C da SG Pedrosa, Goto-Silva L, Gomes IC, Souza LRQ, Ornelas IM, Karmirian K, et al. Non-permissive SARS-CoV-2 infection of neural cells in the developing human brain and neurospheres. *BioRxiv* 2020. 09.11.293951 [Preprint]. Available from: <https://www.biorxiv.org/content/10.1101/2020.09.11.293951v3>.
- [45] Song E, Zhang C, Israelow B, Lu-Culligan A, Prado AV, Skriabine S, et al. Neuroinvasion of SARS-CoV-2 in human and mouse brain. *J Exp Med* 2021;218:e20202135.
- [46] Ou X, Liu Y, Lei X, Li P, Mi D, Ren L, et al. Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. *Nat Commun* 2020;11:1620.
- [47] Kim J, Chung Y, Jun H, Lee N, Seon M, Hee S, et al. Identification of coronavirus isolated from a patient in Korea. *Osong Public Heal Res Perspect* 2020;11:3–7.
- [48] Wang C, Li W, Drabek D, Okba NMA, Haperen R Van, Osterhaus ADME, et al. A human monoclonal antibody blocking SARS-CoV-2 infection. *Nat Commun* 2020;11:1–6.
- [49] Ou KL, Hosseinkhani H. Development of 3D in vitro technology for medical applications. *Int J Mol Sci* 2014;15:17938–62.
- [50] Ravi M, Paramesh V, Kaviya SR, Anuradha E, Paul Solomon FD. 3D cell culture systems: advantages and applications. *J Cell Physiol* 2015;230:16–26.
- [51] Duraffour S, Snoeck R, Krec M, Oord J Van Den, Vos R De, Crance J, et al. Activities of several classes of acyclic nucleoside phosphonates against camelpox virus replication in different cell culture models. *Antimicrob Agents Chemother* 2007;51:4410–9.
- [52] Koban R, Neumann M, Daugs A, Bloch O, Nitsche A. A novel three-dimensional cell culture method enhances antiviral drug screening in primary human cells. *Antivir Res* 2018;150:20–9.
- [53] Rosellini A, Freer G, Quaranta P, Dovero V, Menichini M, Maggi F, et al. Enhanced in vitro virus expression using 3-dimensional cell culture spheroids for infection. *J Virol Methods* 2019;265:99–104.
- [54] Zhang ZZ, Xu HM, Mazza G, Zhang MM, Frenguelli L, Liu QB, et al. Decellularized human liver scaffold-based three-dimensional culture system facilitate hepatitis B virus infection. *J Biomed Res Part A* 2019;107:1744–53.
- [55] Garcez PP, Loiola EC, Da Costa RM, Higa LM, Trindade P, Delvecchio R, et al. Zika virus impairs growth in human neurospheres and brain organoids. *Science* 2016;352:816–8.
- [56] Jr BS, Tencate V, Uprichard SL. Three-dimensional Huh7 cell culture system for the study of Hepatitis C virus infection. *Virol J* 2009;8:1–8.
- [57] Straub TM, Höner K, Orosz-coghlan P, Dohnalkova A, Mayer BK, Bartholomew RA, et al. In vitro cell culture infectivity assay for human noroviruses. *Emerg Infect Dis* 2007;13:396–403.
- [58] Galindo LT, Mundim MTVV, Pinto AS, Chiarantin GMD, Almeida MES, Lamers ML, et al. Chondroitin sulfate impairs neural stem cell migration through ROCK activation. *Mol Neurobiol* 2018;55:3185–95.
- [59] de Melo BAG, Jodat YA, Mehrotra S, Calabrese MA, Kamperman T, Mandal BB, et al. 3D printed cartilage-like tissue constructs with spatially controlled mechanical properties. *Adv Funct Mater* 2019;29:1906330.
- [60] Goodwin TJ, McCarthy M, Cohrs RJ, Kaufer BB. 3D tissue-like assemblies: a novel approach to investigate virus-cell interactions. *Methods* 2015;90:76–84.
- [61] Garcez PP, Nascimento JM, De Vasconcelos JM, Madeiro Da Costa R, Delvecchio R, Trindade P, et al. Zika virus disrupts molecular fingerprinting of human neurospheres. *Sci Rep* 2017;7:1–10.
- [62] Bullen CK, Hogberg HT, Bahadirli-Talbott A, Bishai WR, Hartung T, Keuthan C, et al. Infectability of human BrainSphere neurons suggests neurotropism of SARS-CoV-2. *ALTEX* 2020;37:665–71.
- [63] Rossi G, Manfrin A, Lutolf MP. Progress and potential in organoid research. *Nat Rev Genet* 2018;19:671–87.
- [64] Qian X, Nguyen HN, Jacob F, Song H, Ming GL. Using brain organoids to understand Zika virus-induced microcephaly. *Development* 2017;144:952–7.
- [65] Baktash Y, Madhav A, Collier KE, Randall Glenn. Single particle imaging of polarized hepatoma organoids upon hepatitis C virus infection reveals an ordered and sequential entry process. *Cell Host Microbe* 2018;23:382–94.
- [66] Zhou J, Li C, Zhao G, Chu H, Wang D, Hoi-Ning Yan H, et al. Human intestinal tract serves as an alternative infection route for Middle East respiratory syndrome coronavirus. *Sci Adv* 2017;3:eaao4966.
- [67] Clevers H. COVID-19: organoids go viral. *Nat Rev Mol Cell Biol* 2020;21:335–6.
- [68] Suzuki T, Itoh Y, Sakai Y, Saito A, Okuzaki D, Motooka D, et al. Generation of human bronchial organoids for SARS-CoV-2 research. *BioRxiv* 2020. 05.25.115600 [Preprint]. Available from: <https://www.biorxiv.org/content/10.1101/2020.05.25.115600v2>.
- [69] Han Y, Yang L, Duan X, Duan F, Payant BEN, Yaron TM, et al. Identification of candidate COVID-19 therapeutics using hPSC-derived lung organoids. *BioRxiv* 2020. 05.05.079095 [Preprint]. Available from: <https://www.biorxiv.org/content/10.1101/2020.05.05.079095v1>.
- [70] Peng L, Liu J, Xu W, Luo Q, Chen D, Lei Z, et al. SARS-CoV-2 can be detected in urine, blood, anal swabs, and oropharyngeal swabs specimens. *J Med Virol* 2020;92:1676–80.
- [71] Cholankeril G, Podboy A, Aivaliotis VI, Tarlow B, Pham EA, Spencer S, et al. High prevalence of concurrent gastrointestinal manifestations in patients with SARS-CoV-2: early experience from California. *Gastroenterology* 2020;159:775–7.

- [72] Zhang C, Shi L, Wang FS. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol* 2020;5:428–30.
- [73] Wang Y, Liu S, Liu H, Li W, Lin F, Jiang L, et al. SARS-CoV-2 infection of the liver directly contributes to hepatic impairment in patients with COVID-19. *J Hepatol* 2020;73:807–16.
- [74] Xu L, Liu J, Lu M, Yang D, Zheng X. Liver injury during highly pathogenic human coronavirus infections. *Liver Int* 2020;40:998–1004.
- [75] Zhao B, Ni C, Gao R, Wang Y, Yang L, Wei J, et al. Recapitulation of SARS-CoV-2 infection and cholangiocyte damage with human liver ductal organoids. *Protein Cell* 2020;11:771–5.
- [76] Jacob F, Pather SR, Huang W, Wong SZH, Zhou H, Zhang F, et al. Human pluripotent stem cell-derived neural cells and brain organoids reveal SARS-CoV-2 neurotropism. *BioRxiv* 2020;27:937–50.
- [77] Ramani A, Müller L, Ostermann PN, Gabriel E, Abida-Islam P, Müller-schiffmann A, et al. SARS-CoV-2 targets neurons of 3D human brain organoids. *EMBO J* 2020;39:e106230.
- [78] Makovoz B, Møller R, Eriksen AZ, TenOever BR, Blenkinsop TA. SARS-CoV-2 infection of ocular cells from human adult donor eyes and hESC-derived eye organoids. SSRN 3650574 [Preprint] 2020. Available from: <https://ssrn.com/abstract=3650574>.
- [79] Lancaster MA. Brain organoids get vascularized. *Nat Biotechnol* 2018;36:407–8.
- [80] Mansour AA, Bloyd CW, Li H, Quang D, Johnston S, Parylak SL, et al. An in vivo model of functional and vascularized human brain organoids. *Nat Biotechnol* 2018;36:432–41.
- [81] Vives J, Batlle-Morera L. The challenge of developing human 3D organoids into medicines. *Stem Cell Res Ther* 2020;1:1–4.
- [82] O'Brien FJ. Biomaterials & scaffolds for tissue engineering. *Mater Today* 2011;14:88–95.
- [83] Schwegmann-Weßels C, Herrler G. Sialic acids as receptor determinants for coronaviruses. *Glycoconj J* 2006;23:51–8.
- [84] Milewska A, Zarebski M, Nowak P, Stozek K, Potempa J. Human coronavirus NL63 utilizes heparan sulfate proteoglycans for attachment to target cells. *J Virol* 2014;88(22):13221–30.
- [85] Madu A, Ikenna G, Victor C, Beverley E, Madu IG, Chu VC, et al. Heparan sulfate is a selective attachment factor for the avian coronavirus infectious bronchitis virus beaudette. *BioOne Complet* 2007;51:45–51.
- [86] Zhang YS, Yue K, Aleman J, Mollazadeh-Moghaddam K, Bakht SM, Yang J, et al. 3D bioprinting for tissue and organ fabrication. *Ann Biomed Eng* 2017;45:148–63.
- [87] He Y, Xie M, Gao Q, Fu J. Why choose 3D bioprinting? Part I: a brief introduction of 3D bioprinting for the beginners. *Bio-Design Manuf* 2019;2:221–4.
- [88] Moroni L, Burdick JA, Highley C, Lee SJ, Morimoto Y, Takeuchi S, et al. Biofabrication strategies for 3D in vitro models and regenerative medicine. *Nat Rev Mater* 2018;3:21–37.
- [89] He Y, Gu Z, Xie M, Fu J, Lin H. Why choose 3D bioprinting? Part II: methods and bioprinters. *Bio-Design Manuf* 2020;3:1–4.
- [90] He Y, Nie J, Xie M, Gao Q. Why choose 3D bioprinting? Part III: printing in vitro 3D models for drug screening. *Bio-Design Manuf* 2020;3:160–3.
- [91] Schöneberg J, Lorenzi F De, Theek B, Blaeser A, Rommel D, Kuehne AJC, et al. Engineering biofunctional in vitro vessel models using a multilayer bioprinting technique. *Sci Rep* 2018;8:10430.
- [92] Hiller T, Berg J, Elomaa L, Röhrs V, Ullah I, Schaar K, et al. Generation of a 3D liver model comprising human extracellular matrix in an alginate/gelatin-based bioink by extrusion bioprinting for infection and transduction studies. *Int J Mol Sci* 2018;19:1–17.
- [93] Berg J, Hiller T, Kissner MS, Qazi TH, Duda GN, Hocke AC, et al. Optimization of cell-laden bioinks for 3D bioprinting and efficient infection with influenza A virus. *Sci Rep* 2018;8:1–13.
- [94] Horvath L, Umehara Y, Jud C, Blank F, Petri-Fink A, Rothen-Rutishauser B. Engineering an in vitro air-blood barrier by 3D bioprinting. *Sci Rep* 2015;5:7974.
- [95] Park JY, Ryu H, Lee B, Ha D-H, Ahn M, Kim S, et al. Development of a functional airway-on-a-chip by 3D cell printing. *Biofabrication* 2018;11:015002.
- [96] Lancaster MA, Corsini NS, Wolfinger S, Gustafson EH, Phillips AW, Burkard TR, et al. Guided self-organization and cortical plate formation in human brain organoids. *Nat Biotechnol* 2017;35:659–66.