Lung-On-A-Chip Technologies for Disease Modeling and Drug Development

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Supplementary Issue: Image and Video Acquisition and Processing for Clinical Applications

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ABSTRACT: Animal and two-dimensional cell culture models have had a profound impact on not only lung research but also medical research at large, despite inherent flaws and differences when compared with in vivo and clinical observations. Three-dimensional (3D) tissue models are a natural progression and extension of existing techniques that seek to plug the gaps and mitigate the drawbacks of two-dimensional and animal technologies. In this review, we describe the transition of historic models to contemporary 3D cell and organoid models, the varieties of current 3D cell and tissue culture modalities, the common methods for imaging these models, and finally, the applications of these models and imaging techniques to lung research.

KEYWORDS: 3D culture, lung organoid, microfluidics, microscopy

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Introduction

Laboratory medicine is considered one of the cornerstones of evidence-based medicine with many innovative ideas, devices, and technologies having changed the direction of medicine.[1] One such example is the idea of a model system that provides a platform for studying and testing novel methods in a controlled and replicable environment. Many models in which various aspects of a disease can be reproduced were designed and rigorously tested. These models range from small and large animals, cell and tissue cultures, and the recent advent of tissue-engineered organoids.[1] Recent advances in tissue engineering and biomaterial research have resulted in successful generation of multicellular tissue models that demonstrate authentic functional properties of the corresponding human tissue/organ. These tissue *organoids* include liver organoids with metabolic activity, contracting skeletal and cardiac muscle constructs, gut, kidney, and brain constructs.[2,3] The next ambitious step in this direction was the evolving of organ-on-a-chip model, which involved culturing living cells, in multicellular tissue or organ-level complexity, in a microfluidic device. Such a device allows continuous perfusion of the cells so as to simulate the circulation in the body and provides a stage for in vitro analysis of metabolic, biochemical, and genetic activities of living cells in the context of a functional tissue and organ.[2] This review provides an overview of the current experimental models and their limitations and highlights recent advances that have facilitated the development of improved models of lung tissue function and disease.

Transition of Historic Models to Contemporary 3D Cell Culture and Organoid Model

Animal models. Animals have historic use in medical science, having been used to study anatomy and physiology since 500 BC.[4] Animals and animal models are an integral part of scientific discovery and innumerable advances would not have happened without them. Despite the immense impact animal models have had on medicine, they have failed to replicate many important human diseases. Mouse models have not been successful in aiding the development of a new treatment for type II diabetes, one of the fastest growing diseases worldwide.[5] Similarly, studies in mice regarding severe inflammatory conditions such as trauma, sepsis, and burns have been unsuccessful in replicating the disease pathology, as it occurs in human beings.[6] Many scientific discoveries and therapies have shown promise in the animal model of choice but subsequently failed to replicate the results when translated to human beings.[7] This is due to the huge differences in associated risk factors, course of disease, pathogenesis, chronicity, related pathologies and comorbidities, symptoms, and genetic influences between the species.[8] A systematic review published in 2008 examined the relevance of animal experiments to the

development of human clinical interventions.[9] According to this review, animal models showed the potential to significantly contribute toward the development of human clinical interventions in only 2 cases out of 20. None of the seven cited toxicological reviews could clearly demonstrate the potential of animal models for correctly predicting the human toxicological outcomes. This difference appears to be due to the marked phenotypic interspecies differences, which manifest in changes to susceptibility, etiology, and course of disease, as well as pharmacokinetics and pharmacodynamics of drugs administered.[9]

Cell culture. Another widespread method to study the cellular, molecular, and biochemical aspects of diseases is through cell or tissue culture. Cell culture has been utilized for countless studies and some examples include the study of uncontrolled cell division in cancer research, toxicological studies, chromosomal analysis of womb derived cells, and functional analysis of transformed nerve cells.[10] Cell cultures are typically performed in two dimensions (2D), where the cells of a single type are grown on plastic or glass dishes. The uniformity of traditional cell and tissue culture allows standardized and consistent study of the morphology, genetics, and physiologic response of the particular cell type, lends itself to high-throughput applications, which positions it as a mainstay in the drug development industry, and is relatively inexpensive, which makes it the go-to for laboratory-based research.[10] Besides being more cost effective, the ethical and regulatory considerations pertaining to animal experiments can be circumvented.

Although it has historic use in both commercial and research endeavors and has many positive aspects, 2D cell culture has inherent drawbacks compared with animal models. For example, tissues and organs are made up of multiple cell types, which function as a unit through cellular communication and contact. They constantly give and receive cues from each other, which build into the complex functionality of a whole organ.[11] In basic cases and applications, the simplicity of 2D cell culture is not always an inherent drawback, but in complex studies where cellular output and communication is important, this simplicity does not accurately represent the entire system of interest. Borne out of the need for both inexpensive and complex in vitro models, three-dimensional (3D) cell culture techniques have been developed. 3D cell culture allows the integration of several cell types along with extracellular matrix (ECM) or scaffolding to direct and instruct cells toward specific phenotypes.[12,13] By substituting the glass or plastic dish of 2D culture for 3D tailor-made architecture, researchers gain another arm of control over their studies. Chemotactic gradients can be produced, which mimic in vivo systems of cellular fate regulation and specification.[14] Microarchitectures that are made in the image of in vivo microenvironments can guide cells into the correct orientation. Even the integration of secondary or supportive cell types can drive self-organization that stimulates cells to act more physiologically.[15,16]

The 3D substrate allows massive customization of the ECM to suit the cells of interest. For instance, studies on hydrogels demonstrated that the matrix stiffness and topography contribute to the nature of adhesion, morphogenesis, differentiation, and viability.[17,18] In contrast, 2D cultures provide a static nonphysiological environment, being traditionally performed on glass or plastic surfaces. Another avenue of modification comes from the factors present within the ECM, which are also instrumental in various cellular functions. ECM can be loaded with tissue-specific extracts that include growth factors and other components to aid physiologic cellular function.[19–21] In all, 3D culture allows immense control over the cellular environment that leads to more physiologically accurate modeling.

Varieties of Current 3D Cell and Tissue Culture Modalities

3D culture models are meant to bridge the gap between cell culture and living tissue, and they can be grouped into the following three categories[12,22]:

- organotypic explant cultures,
- cellular spheroids, and
- tissue-engineered models.

Organotypic explant cultures. Organotypic explant cultures rely on whole or partial organ explants grown on a sufficient substrate for maintenance and viability. These models have the benefit of completely recapitulating in vivo microarchitecture and function, but can be expensive to obtain and do not allow the level of control of other 3D systems. Organ explants embedded in collagen gels or cultured on semiporous membranes have been used to produce such explant cultures.[12] Organotypic explant cultures of mouse lung were obtained by explanting lungs from mouse embryos, resulting in moderate development characterized by further branching of the bronchial tract and development of lobules.[23] Precision-cut lung slices (a method of creating microslices of tissue using a vibratome) have been used to study the properties of normal and diseased lungs.[24] Response to allergens, stimuli like hypoxia or bronchodilators, smooth muscle contractility, smoking-related toxicology, and effects of infectious agents have been studied in these lung models.[24] Although organotypic explant cultures make it feasible to study in vivo microenvironments in vitro, they are difficult to procure, troubleshoot, and optimize. The variability of source organs can create experimental variance, and the handling of organs introduces a chance of contamination.

Cellular spheroids. Cellular spheroids are groups of aggregated cells generated by spontaneous cell aggregation, hanging drop technique, or rotating wall vessel cultures. These cell spheroids do not require any external scaffold for

aggregation and instead rely on cellular contact and spontaneous ECM deposition to hold them together. Some methodologies call for an integrated microcarrier to act as an attachment foci and initiate cellular aggregation.[25–27] Cell-only spheroids are limited in size due to the absence of a vasculature, with larger spheroids developing necrotic cores.[28] The use of microcarriers has assisted in the development of larger constructs (even up to 1 cm) wrapped around them to remain viable without signs of necrosis or enhanced apoptosis.[29] Spheroids have been used to study cell metabolism, necrosis, angiogenesis, and adhesion.[28] They are widely used for drug screening and also provide a platform for various electrophysiological and immunohistochemical studies.[28] Epithelial cells, which are often typified by organized and aggregated structure in vivo, can be formed into spheroids using both microcarrier and microcarrier-free techniques.[25,30–32] These aggregates develop physiologic cell–cell contacts, sometimes form lumen, and often secrete mucins characteristic of in vivo tissues.[31] Spheroids share similar characteristics with in vivo tumors, which makes them excellent models used for cancer research.[33,34] Spheroids are extensively used in cancer biology to study invasion, metastasis, antineoplastic drugs, radioresponsiveness, and hypoxic effects.[35] Spheroid-based 3D culture represents a small step from basic 2D culture while still retaining many of the benefits of more involved 3D techniques.

Tissue-engineered models. Tissue engineering applies the principles of biology and engineering to develop functional substitutes of various tissues, which can be used either to replace damaged tissues or for various research purposes in the form of in vitro models. Over time, tissue engineering has revealed the importance of specific microenvironmental cues for cell growth and function. Since 3D cultures are thought to be able to provide these cues, there has been a paradigm shift from 2D cultures to engineered tissues representative of the human body. Human airway epithelium has been modeled using normal human bronchial epithelial cells grown over fetal lung fibroblasts, which are immersed in collagen type 1.[36] This was exposed to air–liquid interface to assist polarization. The model showed ciliogenesis and ciliary motility as well as response to inflammatory cells and chemical and mechanical stress. However, ciliogenesis never reached 100%, mucociliary transport was not uniform over the epithelium, and the level of pseudostratification achieved was not identical to that seen in vivo. Tissue-engineered models allow high levels of tuning and modification. Substrate stiffness, fiber alignment and density, growth factor tethering, cellular content, and more can be matched to in vivo specifications to direct cells into tissue-like organizations.[37–40] These tissue-engineered organoid constructs represent high functioning and highly accurate models for diagnostics and in many organ systems are the leading edge of research technologies. These organoids may be the pinnacle of in vitro generated models, but their complexity makes for time-consuming and expensive experiments.

Lung Organoids

An organoid culture system is essentially a 3D tissue-engineered culture system that accurately replicates the histological and functional aspects of the in vivo tissue. By developing these structures and functions, the organoid emulates the in vivo physiological or pathological environment. It often has multiple types of cells mirroring its counterpart in the body along with constituents of native ECM and is typically miniature or microscale in size.[3] Such cultures are seen as an important avenue to reconstituting organ functions ex vivo. Many of these systems aim to also model multiscale architecture and tissue–tissue interfaces, such as the interface between vascular endothelium and surrounding connective tissue and parenchymal cells, which are crucial to the function of nearly all organs.[3] Additionally, organoid cultures can be exposed to normal mechanical cues, including fluid shear stress, tension, and compression, which influence organ development and function in healthy and diseased tissue.[3] The inclusion of fluid flow also provides opportunities to study how cultured cells interact with circulating blood and immune cells.[2] This strategy has led to the development of *organs-on-a-chip*, which integrates 3D cell culture systems with microfluidic technologies to better mimic the mechanical and circulatory environment in living tissues.

As with other organs, 3D cultures of the lung can be constructed in a number of ways depending principally on the extent to which the structural and functional complexity of the organ is to be mimicked. It has been seen that when primary human alveolar type II cells were cultured in 3D Matrigel™, a commercially available basement membrane, alveolar-like cysts resulted, which are composed of a central lumen surrounded by an epithelial cells monolayer; these formations are a hallmark of in vivo structure.[41] The cells exhibited properties characteristic of differentiated alveolar type II cells (AT II), for instance, surfactant and tubular myelin production, and they were highly polarized. Immortalized, bronchial, epithelial tissue organoids have been devised from human biopsy specimens and demonstrated retention of epithelial morphology. They were positive for markers such as cytokeratins 7, 14, 17, and 19 (epithelial cells) and p63 (stem cells), possessed high levels of $p16^{INK4a}$, and have an intact p53 checkpoint pathway. Microarray gene expression profiling showed clusters of such immortalized bronchial cell lines being distinct from clusters of lung cancer cell lines.[42]

The 3D culture assays of lung organoids using multiple cells and cell lines have also been reported.[43] Fibroblasts or fibroblast-conditioned medium in primary cell cocultures have been shown to enhance the proliferation of primary bronchial epithelial cells compared with bronchial epithelial cell monoculture. When cocultured in Matrigel™ along with a monolayer of IMR90 fetal lung fibroblasts, bronchial epithelial cells formed either cyst-like structures or tubular structures, depending on the microenvironment. Absence of fibroblasts hampered proliferation of bronchial epithelial cells.

Thus, these cells seem to be capable of producing structures that look like branched alveoli under suitable conditions.[43]

Lung organoids can also be derived from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), or primary stem cells that are found throughout the lung.[44,45] While ESCs are primitive cells derived from inner cell mass of the blastocyst, iPSCs are derived from adult cells by reprogramming. Primary/adult stem cells are undifferentiated cells found in a tissue among differentiated cells. In a study describing the generation of fully differentiated airway epithelial cells from murine ECSs, the ESCs could be induced to differentiate into multiple cell types present within the airway epithelium (ciliated, intermediate, basal, and Clara cells), while maintaining cellular ratios of native tissue. The study also reported the appearance of mesenchymal tissue with blood vessels and cartilaginous structures located under the epithelium. In addition, mature cilia, functional cellular junctions, and secretion of CC10 protein by Clara cells were established in the model.[44] In another study, it was shown that a combination of FGF10 with retinoic acid can drive differentiation of human foregut stem cells into lung bud representing cells. This system was used to study cystic fibrosis (CF) by using human foregut stem cells derived from CF patients to model important aspects of the disease in vitro such as defective chloride transport across the cell membrane and reproducing the effects of VX809, a small molecule, which restores the function of CFTR.[45]

Lung organoid platforms facilitate the study of organogenesis, tracking of lung physiology in health and disease, modeling mechanisms of pulmonary disease development, and allow the integration of microfluidics with these organoids to produce complex lung-on-a-chip models.[3]

Microfluidics in 3D Culture

The incorporation of microfluidic devices in 3D cultures has allowed for continuous perfusion of cells in culture.[2] This permits not only long-term study through the use of large media reservoirs and the modeling of fluid shear effects but also connection of modular components through a single circulating system such as several organs-on-a-chip or an array of sensors. Microfluidic culture systems are generally made by soft lithography, although micromolding, microetching, solid object printing, photopolymerization, laser etching, and injection molding can also be used.[2] Multiple organoid systems can be connected by microfluidic channels so that various tissues can communicate with each other and behave in a manner that represents the interactions of organs in the body. Microfluidic designs have been utilized to study the effects of physical, chemical, and electrical stimuli on the cells and tissues—the modularity of microfluidics makes for easy integration of electrical probes, various sensors, and mechanical actuators.[46–48] Cyclical strain or cellular deformities due to fluid shear stresses that many in vivo tissues experience can be reproduced using microfluidics; for instance, a *breathing* lung-on-a-chip was developed by integrating vacuum chambers into the microfluidic module.[3] This same approach was adapted to create small airway-on-a-chip model to study the effects of different proinflammatory factors on epithelial histology, cell secretory functions, as well as ciliary activity mimicking conditions such as asthma and chronic obstructive pulmonary disease (COPD).[49] A microfluidic device in this model was made of polydimethylsiloxane using soft lithography with differentiated mucociliary bronchiolar epithelial cells and endothelial cells seeded on either side of the membrane to mimic the human small airways.[49] Microsensors, which can detect cells and environmental conditions, have been incorporated into these chips to report barrier function, fluid pressure, and cell migration.[2] Microfluidics benefit organoid culture by increasing longevity, easing data collection, and creating a more physiologic environment for cells; these aspects make it an ideal technique for developing complex model cultures.

Microfluidics for lung organoid culture: a lung-on-a-chip. In a report by Huh et al, a multifunctional, microfluidic device was designed to emulate the human alveolar–capillary interface.[3] This device sought to reproduce key structural, functional, and mechanical properties of the alveolar–capillary interface in an effort to create a highly relevant model of lung microphysiology. Human alveolar epithelial cells and human pulmonary microvascular endothelial cells were cultured on the opposite sides of an ECM-coated membrane until they were confluent. The membrane is situated within a microfluidic device with separate channels supplying each side of the membrane, resulting in two separate compartments: one epithelial and one endothelial. After confluence was achieved, the epithelial compartment was exposed to air to create the lung's characteristic air–liquid interface. The microfluidic system was used to manipulate fluid flow and deliver nutrients to the epithelium and endothelium independently. The system also sought to replicate the breathing movements induced dynamic mechanical distortion of the alveolar–capillary interface by incorporating two lateral chambers attached to vacuum pumps. The epithelial layer showed surfactant production along with enhanced electrical resistance and molecular barrier function compared with cultures outside of the microfluidic device.[3] Due to the lung's unique architecture, it is a tissue of interest for microfluidic model development as evidenced by the aforementioned model.

Key parameters to consider while building a lung-on-a-chip model include the following: (i) bilayered channel design separated by a thin membrane (to mimic alveolar/epithelial' and endothelial compartments), (ii) incorporation of combined solid and fluid stresses, (iii) coculture of both epithelial and endothelial cells, and (iv) the integration of biological sensors to detect real-time changes in barrier behavior.[50]

Real-time monitoring of barrier function for lung tissue culture studies. Barrier integrity is imperative to maintain as the barrier between liquid and air generates the gas exchange characteristics of the lung. Without a complete

barrier, the model can leak air or liquid from one side to the other, which destroys the interplay between epithelial and endothelial cells. The barrier can be characterized in vitro by various methods such as immunostaining for proteins characteristic of tight junction proteins (occludin, ZO-1, and ZO-2),[51] electron microscopy of transmembrane fibrils, radiolabeled paracellular permeability markers, and transepithelial/endothelial electrical resistance (TEER; Fig. 1A).[52] Thus, these methods make it possible to measure the integrity of the barrier in response to various stimuli. TEER measures electrical resistance through a cellular monolayer and is a very sensitive, accurate, and reliable method to determine the integrity and permeability of the monolayer.[53] Although TEER measurements are less representative of the long-term character of the tissue than radiolabeled permeability markers or tight junction protein assays, it enables an instantaneous and live cell quantification of tightness of a cell layer.[54] In microfluidic lung models, TEER measurements are effective due to real-time capability and, when performed correctly, result in negligible cellular damage.[3,52]

Imaging Methods for 3D Cell Biology

Imaging techniques are an integral aspect of contemporary research. Given that tissues are made of microscopic cells and those cells are made from nanoscopic proteins, lipids, and molecules, microscopic imagery is essential to studying cellular dynamics and tissue architecture. Clinical imaging allows the resolution of tissue makeup and cellular orientation;[55] with our massive database of healthy and diseased tissue, it is possible to make diagnoses based purely on qualitative comparison of biopsied and imaged tissue to historical images. Unfortunately, 3D cell cultures are difficult to image; they are dense, optically opaque, and large, which makes light penetration almost impossible. However, in the past several decades, techniques for resolving the structure and architecture of 3D constructs have been developed and refined.[56,57] These modalities range from adaptations from existing techniques to completely novel systems built around imaging 3D tissue constructs.[58]

Confocal laser scanning microscopy. Presently, confocal fluorescence microscopy is the most widely used advanced imaging technology for 3D models. Confocal laser scanning microscopy relies on exciting fluorophores with a tunable laser and exploiting the resultant focused light to generate high-resolution images.[58] A pinhole is situated between the sensor and the lens assembly; this pinhole is only wide enough to allow focused light to reach the sensor, whereas out-of-focus light is blocked by the pinhole housing. This allows extremely shallow depths of field to be resolved with little to no noise or background while using a single wavelength laser to isolate only a specific fluorophore of the 3D construct. In addition, automated stages can be used to create image *stacks*, where a different confocal image is generated at periodic depths throughout the construct; 3D renderings can be generated from these stacks using interpolation and reconstruction software (Fig. 1B). This modality can be used for relatively thicker sample both in reflectance and fluorescence mode.[58] However, in confocal imaging, there is a risk of photobleaching (and phototoxicity for live samples) if used for extended periods; the entire construct is exposed to laser energy, and thus, areas that are not being actively imaged will be exposed to high levels of light.[59]

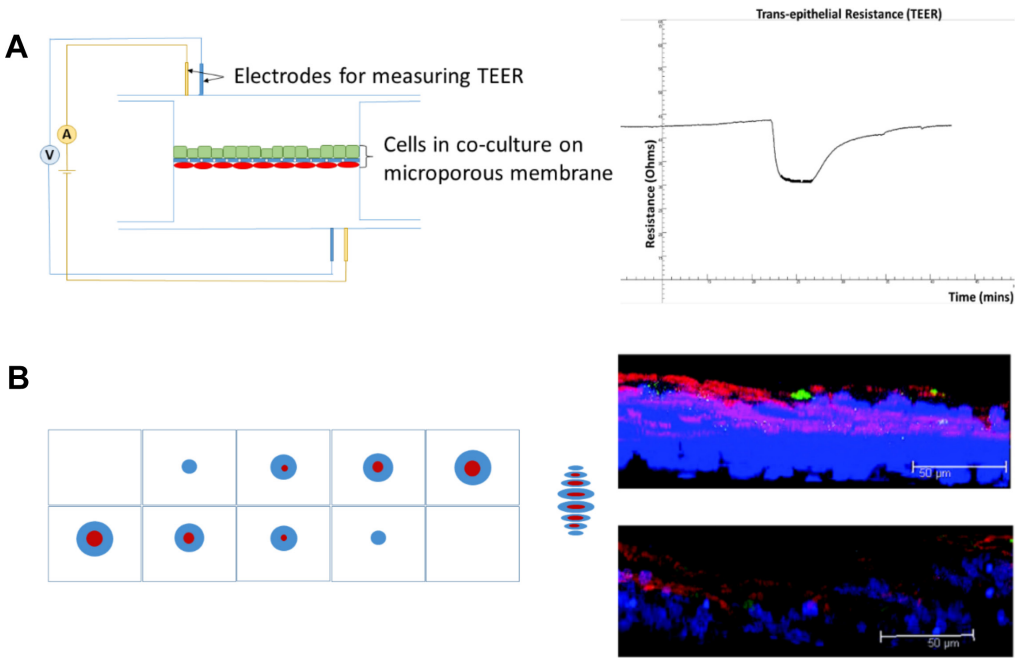

Figure 1. (*Continued*)

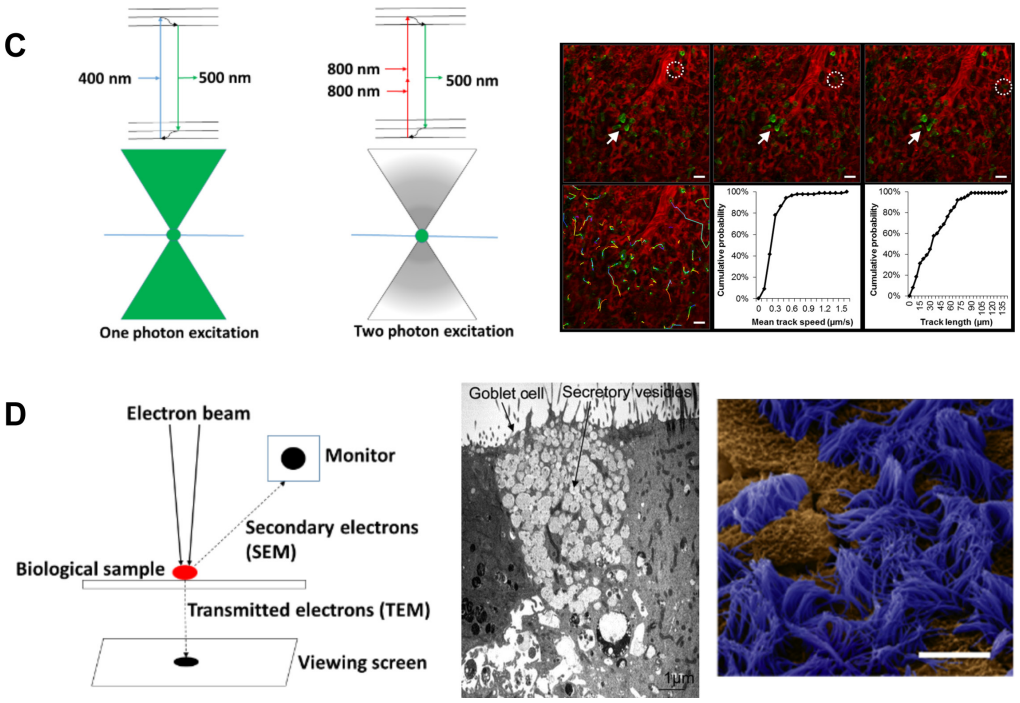

Figure 1. (**A**) TEER provides a platform for automated impedance-based monitoring of cells by analyzing cellular permeability, which reflects the barrier function caused by normal tight junction epithelial connections. TEER can be used to analyze drug effects such as the opening of chloride or sodium ion channels, as well as potential toxicity, resulting in the breakdown of barrier function. Forskolin-induced CFTR ion channel opening is shown as an example (unpublished data). (**B**) Confocal microscopy enables us to collect gallery of sequential image *stacks*, where a different confocal image is generated at periodic depths throughout the construct. 3D renderings or *Z stacks* can be generated from these stacks using interpolation and reconstruction software. Z stack of a nonasthmatic (top) and asthmatic (bottom) pediatric bronchial epithelium at day 28 of culture growing at air–liquid interphase is shown as an example. (Reproduced with permission from Ref. 60.) (**C**) As opposed to one-photon microscopes, two-photon microscopes use two photons of longer wavelength for excitation. Longer wavelength accounts for greater tissue penetration. With two-photon microscopy, only at the focal point are the photons sufficiently dense to interact simultaneously with a fluorophore so fluorescence is limited to the focal region. Two-photon microscopy has been used to track the movement of neutrophils (in green) along pulmonary vessels (red). (Reproduced with permission from Ref. 70.) (**D**) For TEM, the electron beam passes through the specimen and produces an image when the transmitted electrons hit a fluorescent screen at the base. For SEM, the electron beam scans the specimen systematically so that the reflected secondary electrons are registered by a detector. TEM has been used to establish the complete differentiation of pediatric bronchial epithelial air-liquid interface (ALI) cultures into a well-differentiated and functioning epithelium, and SEM has been used to identify differentiated bronchial epithelium with ciliated (cilia in blue-pseudocolored) as well as nonciliated cells (brown-pseudocolored) formed on the microfluidic airway-on-chip model. (Reproduced with permission from Refs. 49 and 60.)

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Confocal microscopy is commonly used in 3D cell and tissue models to analyze the localization and quantity of different cell types, organelles, or specific proteins within the models. This can be done by labeling the cells with fluorescent dyes. For example, when a 3D model of pediatric bronchial epithelium was developed from cells of asthmatic and nonasthmatic children, computational analysis of confocal microscopy images from each of the models demonstrated that the percentage of goblet cells and ciliated cells varied between the two groups.[60] In a study reporting the generation of multiciliated airway epithelial cells from iPSCs, the localization of Forkhead box protein J1 (FOXJ1), a transcription factor associated with ciliogenesis was confirmed by confocal microscopy, which also revealed that most ciliated cells are in the early stages of multiciliogenesis after 28 days in air–liquid interphase.[51] Various articles studying the potential for the use of human and avian lung slices in recapitulating pulmonary infection caused by different bacteria and virus have used confocal microscopy to visualize the infected cells of the lung slices. Studying infective process of cells and host–pathogen interactions may be possible in live lung slices, especially by time-lapse confocal microscopy.[24]

Two-photon and multiphoton microscopy. Two-photon and multiphoton imaging exploits photonic strength to mitigate the effects of photobleaching and increase depth range.[58] The modality utilizes two or more streams of photons, which are absorbed almost simultaneously by the fluorophore and a single photon in the visible range is emitted—neither photon stream is of high enough strength to elicit emission but together they generate fluorophore excitation. The photon absorption is restricted within the focal volume, resulting in high spatial resolution as well as reduced photobleaching and phototoxicity (Fig. 1C). Near-infrared wavelengths can be used to allow greater depth penetration.[58] These features help in imaging thick or highly scattering tissue. This type of microscope provides high-resolution imaging and allows a much greater depth penetration than standard confocal microscopy, which makes multiphoton microscopy good for imaging large specimens.[24,58]

Two-photon microscopy has been used to study airway reactivity and assess the presence and distribution of collagen in the ECM without using dyes.[24] Both ECM and intracellular events such as exocytosis could be visualized together, the former as described above and the latter stained by fluorescence dye, by collecting the emitted fluorescence. Confocal and two-photon microscopy were also used in a study to image acellular murine lung matrix in sequential sections and assess the efficacy of the decellularization process as evidenced by lack of cells or cell membranes and scarcity of nuclear material.[61] 3D reconstruction of the sequential segments showed the relative makeup of the acellular lung. Imaging was performed at different depths and showed preservation of basic architecture, presence of type I fibrillar collagen, and absence of intact cells present within the fully decellularized tissue. Multiphoton microscopy was used to visualize the different cell interactions in engineered bronchial tissue consisting of human lung fibroblast cell line, epithelial cells (expressing tdTomato), and dendritic cells (labeled with carboxyfluorescein diacetate succinimidyl ester).[62] Live imaging analysis confirmed the presence of dendritic cells at the apical side of the epithelial cell layer closely associated with the latter. Fibroblast collagen matrix rich in ECM proteins, a compact continuous stratified squamous epithelium on top of an intact basement membrane, were also visualized. Two- and multiphoton microscopy images typically take longer to generate, but this modality allows versatile imaging (both fluorescent and second harmonic), high penetration (due to near-infrared wavelengths), and informative postprocessing (3D reconstruction and image segmentation), thus generating data with great accuracy.[58,61,62]

Transmission electron microscopy. In transmission electron microscopy (TEM), a high-energy beam of electrons is passed through a very thin sample, and the interactions between the electrons and the atoms is used to observe the ultrastructure of cells and ECM.[63] Resolutions attainable from TEM images are much better than that from a light microscope because the wavelength of an electron is much smaller than light (Fig. 1D). TEM has been used quite extensively for imaging organoids. Ultrastructural analysis of the 3D cell aggregates of undifferentiated mouse ESCs (mESCs) encapsulated in alginate beads and grown in different media in rotating vessel bioreactor was performed using TEM.[64] The study compared the different outcomes of using different growth media for the mESCs. It confirmed the differentiation of the mESC to airway epithelium as early as day 7 in the group growing on conditioned media as evidenced by the presence of type II pneumocytes with abundant lamellar bodies and microvilli in TEM images. On the other hand, the control group, growing on maintenance media, did not show such typical pneumocyte-specific organelles, rather they contained a large number of apoptotic cells characterized by condensed nuclei.[64] TEM was also used to study the effect of dexamethasone on tight junctions and adherens junctions of lung epithelial cells grown in coculture with pulmonary microvasculature endothelial cells.[65] In the presence of dexamethasone, reorganization of the apical junction was seen by TEM. This was absent in the non-dexamethasone-stimulated group, which showed nonspecific focal separation between neighboring cells.[65] Biopsy specimens of engineered bronchial mucosa consisting of human bronchial epithelial cells and fibroblasts from normal and asthmatic donors were imaged by TEM.[60] Presence of microvilli and rudimentary cilia was seen in both cases.[60] TEM is a very useful modality of imaging 3D cultures but is limited by its high cost and longer, more complex sample preparation procedures, including sample fixation that prohibits live cell imaging.

Scanning electron microscopy. Scanning electron microscopy (SEM) is becoming a popular method for the visualization of topography and composition cultures grown in 3D cultures. High-resolution images are generated by scanning samples with high-energy electron beams.[63] The sample must be fixed and dried using either lyophilization or critical point drying before being coated in a thin layer of metal particles (sputter coating). The SEM then utilizes a high-energy stream of electrons to scan the sample; the electrons collide with the metal ions and cause electron emission, which is imaged by the sensor (Fig. 1D). In a study describing the effect of dexamethasone on tight junctions and adherens junctions of lung epithelial cells grown in coculture with pulmonary microvasculature endothelial cells, SEM imaging in nontreated samples showed the appearance of *hills and valleys* probably indicating a multilayered structure of epithelial cells.[65] The confluent monolayer established by day 7 of cocultivation was disrupted by day 14 in this group. This was seen as a totally disorganized surface with reduced microvilli and cytoplasmic pseudopodia encroaching on adjacent cells. Even the endothelial layer was disrupted by day 14. In contrast, the dexamethasone-treated group showed maintenance of an evenly organized epithelial monolayer with intact apical junctions and normal microvilli throughout the entire period. Even the endothelial layer retained the

cobblestone morphology throughout, showing flattened cells with protruding nuclei and wide cytoplasmic expansions. In a study describing the generation of airway epithelial cells from murine ESCs, SEM along with quantitative histologic examination and immunohistochemistry were used to characterize the bioengineered epithelium.[44] Computer-assisted SEM was used to calculate the percentage of the area of culture covered by ciliated epithelium. SEM analysis found that most of the ESC cultures after differentiation was covered by ciliated patches, interspersed with Clara cells, intermediate cells, and undifferentiated cells.[49] The limitations of SEM are similar to TEM in addition to offering somewhat lower resolution than TEM, but in turn, it allows the imaging of larger samples and can be used to generate images of scaffolds, hydrogels, and biomaterials used for 3D culture.

Time-lapse imaging. Time-lapse imaging entails using any type of live imaging to take images at a regular frequency. The images can then be played sequentially in video format to visualize long-term changes over a short course of video; for instance, an image of cells cultured on a plastic dish can be performed once in every 10 minutes for 3 days, resulting in 432 frames—or around 18 seconds—of video. Time-lapse of lung slices was used to study the contraction and relaxation of airways and blood vessels in precision-cut lung slices by digital time-lapse microscopy. This type of imaging can also be used for screening new drugs such as novel bronchodilators on lung slices by tracking the airway contraction and relaxation as evidenced by the change in area of airway lumen. This provides a platform for performing automated bioassay using lung slices.[24] Time-lapse imaging can also determine ciliary beat frequency in undisturbed lung slices of the lower airways, which was around 20–25 Hz in contrast to the same study performed in the nasal mucosa, or upper airways, which showed a ciliary beat frequency to be 5–10 Hz. It was also shown that the beat frequency in the former was unaffected by adenosine triphosphate (ATP) or adenylyl cyclase but was increased by phosphodiesterase 4 inhibitors. Time-lapse phase-contrast imaging is limited by tissue thickness and density. Thicker or denser tissues are unsuitable because of the loss of details.[24]

Imaging is an indispensable part of studying 3D cultures due to their unique characteristics, such as containing multiple cell types, use of scaffolds, and increased tissue thickness/density. 3D cultures and constructs present challenges that make them almost impossible to image on the normal instruments used for 2D cultures. 3D imaging modalities are disparate and each have their own sets of benefits and drawbacks, thus it is important to tailor the imaging mode to the experimental plan to mitigate shortcomings and accentuate advantages.

Applications of Lung Organoids

Lung cancer research. 3D organotypic culture models have great potential for cancer precision medicine research and therapeutics. It can be used for a variety of purposes such as oncogene modeling, screening of chemotherapeutic drugs in an individualized manner, and studying chemosensitivity of drug candidates.

Anchorage-independent assays using 3D spheroids using tumor cells have been developed for drug discovery. Drug screening on these spheroids as high-throughput screening in 96-well plate assays are popular. Though most cells form spheroids spontaneously, even those that do not do so can be induced to form spheroids by growing them with spheroid-forming nonclonogenic feeder cells in coculture.[66] For example, one such coculture model can be prepared using non-small-cell lung cancer cell lines and lung fibroblasts. Up to three different cell types have been used for constructing a single spheroid (lung tumor cells, fibroblasts, and endothelial cells).[67] However, there has been no proof of microcapillary functionality.[67] It has been seen that such 3D spheroid culture demonstrated altered cellular response to drugs and growth factors, suggestive of a more accurate representation of the natural microenvironment of the tumor than classical 2D culture. Such multicellular tumor spheroids provide a very useful model for reproducing cellular heterogeneity and providing more complete appraisal of tumor response to various therapeutic strategies such as chemotherapy and radiotherapy. Microfluidic chips with these cocultures are designed to mimic the circulation of nutrients in and metabolic waste out of the cultures by continuous media supply. The chemoresistive testing of spheroids of pleural mesothelioma cancer cells revealed that growth inhibitory concentration of cisplatin showed higher concentration in perfused tumor spheroids compared with spheroids cultured under static conditions. Non-small-cell lung cancer has also been seen to acquire epithelial–mesenchymal transition and cancer stem cell-like phenotypes when cultured in 3D tumor spheroids under specific conditions.[67] There is a large body of literature that extols the virtues of 3D cell culture, and there is a growing understanding of the role of the tumor microenvironment in cancer progression. To properly model and study cancer, it is imperative to mimic the native microenvironment and 3D culture techniques, coupled with microfluidics, promise the means to do just that.

Infectious disease research. Artificial culture media, animal models, or cell lines have been used for growing and studying bacteria. Artificial culture media often supply specific nutrients required for the bacterial growth in much higher concentration than under physiological conditions. So the bacterial growth parameters under culture conditions do not adequately represent those within the human body. Pharmacodynamic studies in artificial culture media cannot be expected to give the accurate results as the bacterial concentration and growth kinetics do not match clinical observations. Bacterial cultures are fickle and require specific microenvironment to grow at the rate seen in physiologic settings: contemporary models of bacterial growth typically demonstrate unsustainable proliferation.

A strategy for bacterial cell culture is to produce an in vitro environment similar to the zones bacteria inhabit in the body. To faithfully recapitulate the biological environment, a human lung tissue model was established for *Mycobacterium tuberculosis*. This lung tissue model was established using human primary macrophages and monocytes along with lung epithelial pneumocytes' cell lines and fibroblasts cultured on a collagen matrix. This system was then exposed to human macrophages infected with mycobacteria (mtb). Granuloma formation, role of ESAT-6 (secreted by the type VII secretion system of virulent strains) secretion in granuloma formation, and tissue necrosis could be replicated in this model. Mtb-infected macrophages along with uninfected PKH26-labeled monocytes were introduced together at a ratio of 1:5. Confocal microscopy at D7 and D10 showed that monocytes formed clusters at the site of infection in the presence of virulent Mtb (H37Rv) infection, indicating early granuloma formation. The aggregates of monocytes could be quantified by the intensity of fluorescence emitted. This model demonstrated both a proof of concept for bacterial culture in a 3D lung model and also presented new results about bacterial-lung cell dynamics.

Inflammatory diseases of the lung. 3D models of asthmatic and nonasthmatic bronchial epithelium were developed by growing primary cells from asthmatic and nonasthmatic sources at air–liquid interface. The aim was to develop a tool for studying the various mechanisms at play during the pathogenesis of asthma.[60] The differences between the two models were assessed, and it was found that the asthmatic epithelium contained more goblet cells and less ciliated cells than its normal counterpart. The amount of prostaglandin E2, interleukin-6 (IL-6), and IL-8 was similar in both cases. Similarly, in the small airway-on-a-chip model, treatment with IL-13, which plays a pivotal role in asthma, was associated with increase in the number of goblet cells, decrease in cilia beating frequency, and higher production and secretion of proinflammatory cytokines (measured in the effluent fluid in the vascular compartment).[49] This model was also adapted for studying COPD by using differentiated COPD epithelium and pulmonary microvascular endothelium on the chip. When this chip was stimulated to mimic inflammation by different compounds, there was an upregulation of IL-8 production and M-CSF secretion. This effect was not seen in chips with normal lung epithelium. The study also suggested that M-CSF could be used as a biomarker for viral infection-induced COPD exacerbation since it is upregulated only in the presence of viral mimic factors.[49] Asthma and COPD are conditions of high prevalence. They can be exacerbated by toxins and pollutants in the air that can cause a patient a great deal of discomfort. 3D models that elucidate the mechanics of airborne toxins on such inflammatory epithelia are an excellent method for developing new therapies and treating these conditions.

Drug toxicity testing and drug development. A major prospect for many 3D tissue models are drug discovery applications. Current pharmaceutical pipelines rely on 2D cultures before moving directly into animal studies. 3D cultures can serve as another, highly predictive method for narrowing the pool of candidate drugs. One drug development study aimed to model IL-2-induced drug toxicity in cancer patients, resulting in pulmonary edema. This toxicity was studied in a lung-on-a-chip model, which showed that the enhanced opening of the intercellular junctions of the alveolar epithelium and endothelium of lung capillaries was a result of mechanical forces during respiratory movements acting synergistically with IL-2. In fact, the mechanical forces acting along with IL-2 caused threefold increase of tissue permeability over eight hours compared with IL-2 alone. It was also found that this IL-2 increased vascular leak could be completely blocked by coadministration of angiopoietin-1. Furthermore, since mechanical strain is said to activate TRPV4 channels thus increasing exudation of fluids from the capillaries of the lung, a new TRPV4 inhibitor was tested on this model. The compound showed promising effect by inhibiting fluid leak caused by IL-2 administration along with 10% cyclic mechanical strain. Another study described the use of microfluidic chip-based model to develop a high-throughput, efficient, and accurate drug-screening platform. It was used to screen anticancer drugs used to treat malignancies of the lung. Single and combined chemotherapeutic regimens were tested to demonstrate the scope for individualized therapy. The study revealed the new model to be highly sensitive, less time consuming, and utilizing less amounts of sample and reagent.[68] The asthma and COPD airway-on-a-chip model was used to validate reports that tofacitinib, a drug used for rheumatoid arthritis, could suppress lung inflammation where dexamethasone is ineffective. It also showed that bromodomain-containing protein 4 (BRD4) inhibitor could inhibit lung inflammation by inhibiting neutrophil adhesion under dynamic flow conditions by almost 75%.[49] Lung-on-a-chip models can be used to explore the mechanics of particular diseases and make informed choices about drug development as in the case of the TRPV4 inhibitor. Models can also be using a high-throughput fashion, slotting directly into the drug development pipeline. Integration into the pharmaceutical industry will decrease the cost and time of drug discovery, while increasing the number of candidate drugs making it to market.

Personalized medicine. Models using patient-derived cells can be used for personalized screening and therapies requiring just a biopsy from the patient. CF, an autosomal recessive monogenetic disorder, is caused by mutations to the CF transmembrane conductance regulator. Specific CFTR pharmacotherapy, tailored for individual CFTR mutations, and better in vitro assays to aid selection of the best therapeutic approach for an individual basis are being developed. One such approach is designing patient-specific epithelial organoids. Reprogrammed fully differentiated tracheal and bronchial epithelial cells can attain a proliferative state.[69]

The epithelial cells, derived from biopsies or cryopreserved tissues, are grown in the presence of Rho-kinase inhibitor over a feeder layer of fibroblasts. These reprogrammed cells can maintain their epigenetic memory, be expanded, and upon removal of the feeders and the inhibitor, revert to their original differentiated phenotype. They can be used to form spherical lung organoids or as monolayers of epithelial cells on transwells at air–liquid interface. It is possible to use these for devising forskolin-induced swelling assays for monitoring CFTR activity, measuring transepithelial current, and also to study the effects of CFTR modulators. Forskolin induces minimal swelling in organoids of human subjects with CF or in mice carrying the CFTR F508del mutation as opposed to rapid swelling of organoids derived from healthy controls. Swelling is not seen in any CFTR-deficient organoid. Outside of CF, disease-on-a-chip models can be constructed for cancer or asthma. Personalized models can aid in the development of personalized drug regimens tailored specifically to each patient.

Conclusion

3D organotypic cultures of the lung are a promising way of studying various physiological and pathological processes, occurring in the respiratory system. The methods for producing 3D lung organoids and cultures span the gamut of 3D techniques. Lung slices can be excised and cultured long term in vitro, but require lung tissue and may not be consistent across samples. Spheroids can be produced using lung epithelial cells, but often do not include sophisticated tailoring of the ECM microenvironment. Tissue-engineered models require cells, scaffold, and proteins associated with the microenvironment; these represent the pinnacle of 3D culture techniques and often integrate spheroids and slice models. The most advanced of the tissue-engineered models are called organoids, which recapitulate the histological structures and function of native tissue. Although these models require extensive hands-on manipulation and fabrication, they generate most physiologically relevant results among 3D models. Integration of organoid models with microfluidics produces an organ-on-a-chip; these models not only mimic the microenvironment of native tissue but can also replicate mechanical aspects such as the air–liquid interface in the lung.

As varied as 3D culture techniques are the imaging techniques used to capture them. Confocal imaging using both laser scanning and multiphoton excitation allow high resolution and moderate penetration of 3D tissues. These modalities allow both fluorescent and second-harmonic generation imaging, in addition to standard transmission microscopy. The electron-based imaging modalities, such as SEM and TEM, are different in result but similar in design as they both rely on electron beams instead of light. These techniques cannot penetrate dense or thick tissue, but they give unparalleled views on ultrastructure, in the case of TEM, and extremely high-resolution images of whole constructs, in the case of SEM. 3D constructs require careful consideration when it comes to imaging, and each imaging technique plays a different role in organoid research and development.

In all, in vitro lung modeling is a thriving and rapidly evolving field. It proves to change the way we develop drugs, study cancer, and other diseases. The lung is a unique organ; it has a unique mechanical environment and a unique cellular composition. Although 3D models are gaining traction quickly and show drastic improvement over 2D systems, they still fall short of full, physiologic imitation. Current models are still in their infancy but can already replicate the healthy and disease state, recapitulate the bacterial-lung cell dynamics, simulate the mechanical strain of breathing, and even be used to incorporate patient-derived cells to produce a personalized lung-on-a-chip.

Author Contributions

Wrote the first draft of the manuscript: DK, MD, DVY. Contributed to the writing of the manuscript: DK, MD, DVY, SVM. Agree with the manuscript results and conclusions: DK, MD, DVY, AA, SVM. Jointly developed the structure and arguments for the paper: DK, MD, DVY, AA, SVM. Made critical revisions and approved final version: DK, MD, DVY, AA, SVM. All authors reviewed and approved of the final manuscript.

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