Beyond the Barriers: Impedance-based quality control of *in vitro* lung models

T.H. Phan[1], H. Shi[2,3], C.E. Denes[4], A.J. Cole[5,6], E. Geervliet[7], Y. Wang[28], Y.Y. Cheng[3,9], D. Hesselson[5,6], S.H. Roelofs[7], G.G. Neely[4,5], J.H Jang[10], W. Chrzanowski[1]

[1]The University of Sydney, Sydney Nano Institute, Faculty of Medicine and Health, Sydney School of Pharmacy, Pharmacy and Bank Building A15, Camperdown, NSW 2006, Australia. [2]Burns Research and Reconstructive Surgery, ANZAC Research Institute, Concord Hospital, University of Sydney, Sydney, Australia. [3]Asbestos Disease Research Institute, Concord Hospital, Sydney, Australia. [4]The Dr. John and Anne Chong Lab for Functional Genomics, Charles Perkins Centre and School of Life & Environmental Sciences, The University of Sydney, Sydney, NSW 2006, Australia. [5]Centenary Institute, The University of Sydney, Sydney, NSW 2006, Australia.y Institute, The University of Sydney, Sydney, NSW 2006, Australia. [6]Faculty of Medicine and Health, The University of Sydney, Sydney, NSW 2006, Australia. [7]Locsense B.V., Locsense B.V., Enschede, The Netherlands. [8]Jiangsu Provincial Engineering Research Centre of TCM External Medication Development and Application, Nanjing University of Chinese Medicine, Nanjing, China. [9]Institute for Biomedical Materials and Devices (IBMD), School of Mathematical and Physical Sciences, Faculty of Science, University of Technology Sydney, Broadway, NSW 2007, Australia. [10]Department of Biochemistry, College of Medicine, Inha University, Incheon 400-712, South Korea. authors

Why an *in vitro* lung model?

Chronic pulmonary diseases are a high burden on the healthcare system and on the quality of life (QoL) of its patients. As the third leading cause of death, chronic respiratory diseases have a tremendous mortality rate [1]. Moreover, with a prevalence of 300 million people worldwide, pulmonary diseases are not only mortal but also highly prevalent [2]. The QoL of these 300 million patients is also significantly decreased and deteriorates with the progression of the disease [3]. While this emphasizes the burden of pulmonary diseases, adequate treatment is lacking. Current therapies focus only on reducing the symptoms and management of the disease, rather than curing [4], [5]. This is due to the difficulty of finding appropriate lung mimics to test pulmonary treatments [6].

While in many biomedical fields, animal models are expectable models for therapeutic compound testing, *in vivo* models do have drawbacks, especially in the field of pulmonary diseases. First of all, the practice of animal testing is a subject of ethical debate. Additionally, animal testing has demonstrated inadequate predictability when translating results to clinical trials [7]. This discrepancy is particularly evident in the field of lung pathophysiology, where substantial differences exist between animal lungs and those in humans. For example, while human pathogens exhibit a preference for infecting human cells, they do not replicate in animal model, nor do they cause any harm [8].

The method of delivery stands as a crucial determinant of treatment effectiveness. Without a suitable means of delivering substances to the lungs or other targeted areas in the body, the real-world applications of these treatments are constrained [9]. Hence, a key focus in biotechnology, toxicology, pharmaceuticals, and medical research is the exploration of alternatives to animal models. Significant advancements have been achieved in both academia and industry, with notable developments such as organ-on-a-chip, organoids, and 3D bioprinted organ-like structures.

Strategies like the utilization of transwells have been implemented to establish larger scale 3D lung models. These models are typically maintained under static culture conditions. This implies that the dynamic nature of the human lungs/body and the progression of diseases are not accurately mimicked. Additionally, it becomes challenging to interrogate real-time cellular responses within these systems [10].

In summary, there is an urgent unmet need for a representable *in vitro* lung model.

Differences between healthy and diseased lungs

To find therapeutic targets and test therapeutic agents *in vitro*, disease understanding and key differences of a healthy and a diseased lung are essential. First of all, the lung serves as the barrier to prevent infectious, cytotoxic and other harmful compounds entering the bloodstream. Creating a strong and discriminating barrier is of vital importance for a healthy lung [11]. Furthermore, the respiratory mucosa consists of a pool of differentiated cells. Each cell type plays a distinct role in the complex physiology and defense mechanisms of the pulmonary system. The five main cell types are: ciliated cells, goblet cells, basal cells, club cells, and neuroendocrine cells. Ciliated cells, the most abundant cell type in a healthy lung, facilitate the mucociliary escalator, a key defense mechanism in the lungs. Goblet cells produce mucus to trap particles, while cilia of the ciliated cells move the material towards the pharynx for expulsion. Basal cells serve as stem cells, supporting the maintenance of ciliated and goblet cells. Club cells have been attributed several protective roles, including participating in airway repair after injury, secreting anti-inflammatory and immunomodulatory proteins, and aiding in detoxification processes. Neuroendocrine cells, also called Kulchitsky cells, can secrete various factors, including catecholamines and polypeptide hormones [12], [13]. However, when the lung is affected by either acute or chronic diseases such as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), acute respiratory distress syndrome (ARDS), etc., the characteristic features of a healthy lung are compromised or lost.

The barrier function of a diseased lung is impaired, and the measurable resistance of this barrier is significantly reduced [14]–[16]. Furthermore, at cellular level, lung injury has major consequences. An

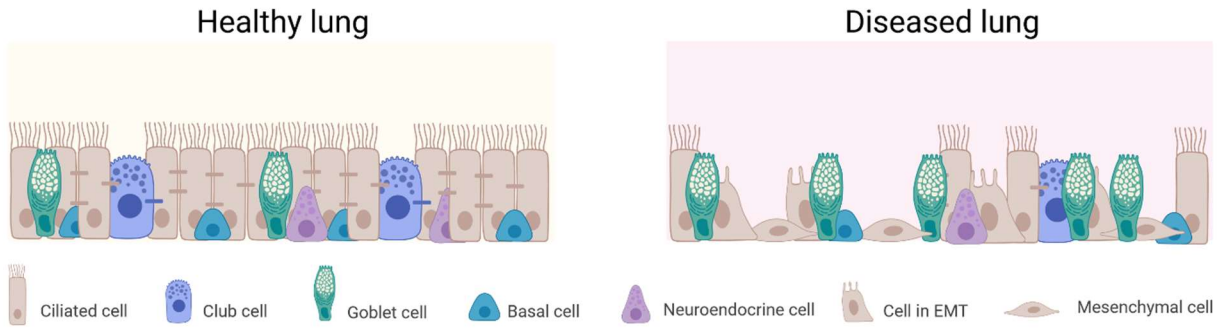

Figure 1. *Difference in healthy and diseased lung epithelium. A healthy lung consists of fully differentiated cells, mainly composed of ciliated cells, with strong tight junctions, inducing an intact barrier. In a diseased lung, goblet cells are overtaking the ciliated cell population and epithelial-mesenchymal transition (EMT) leads to loss of ciliated cells and strong tight junctions, leading to an impaired barrier.*

important process during wound healing and tissue repair is Epithelial–mesenchymal transition (EMT) [17]. EMT is defined as a process in which epithelial cells gradually acquire a mesenchymal (fibroblast-like) cell phenotype [18]. A diseased lung is characterized by loss of ciliated cells, increase of the goblet cell population and excessive EMT, leading to low cell complexity and differentiation [17], [18]. The key differences between a healthy and diseased lung are depicted in **Figure 1.**

In summary, to fabricate a healthy and diseased lung *in vitro*, key characteristics such as barrier function and cell differentiation should be highlighted.

Measuring is knowing

In order to manufacture a representative lung model *in vitro* the key characteristics above –healthy lung model with intact barrier and differentiated cells, a diseased model with an impaired barrier and mostly mesenchymal cells- should be met. Quality control (QC) is therefore as important as the model itself. The QC should (1) be able to measure the barrier function of the lung model without disrupting the barrier itself (2) be able to continuously monitor the barrier function (3) be able to distinguish non-differentiated cells and fully differentiated cells (measure cell capacitance).

To measure the barrier function of a cell layer, different techniques have been developed, such as freeze-fracture electron microscopy possibly combined with immunostaining for tight junction proteins, paracellular tracing of labeled compounds and transepithelial/transendothelial electrical resistance (TEER) measurement, possibly combined with frequency-dependent impedance spectroscopy [19].

Freeze-fracture electron microscopy

Freeze-fracture electron microscopy combined with immunostaining for tight junction proteins is an excellent barrier function technique. Here, the barrier is snap-frozen, broken apart and imaged using an electron microscope, which provides a wealth of structural information [20]. The addition of immunostaining for tight junction proteins adds specific information of the type and location of tight junctions. Together, this gives a well-defined image of the barrier function [21]. However, due to the freezing, this technique is an end-point measurement with destructive characteristics, making it unfit for continuous QC.

Paracellular tracing

Another technique is the paracellular tracing of a labeled compound. This compound is subjected to several requirements. The ideal drug for use should be metabolically inactive, non-toxic within the prescribed doses, free from binding to other molecules like proteins in plasma or tissues. It should be available in various molecular sizes, visible across a spectrum from the naked eye to the electron microscopic level, and reliably quantifiable. Even though many markers have been manufactured, no marker fulfills all criteria, making this technique also unfit [22].

Electrical impedance spectroscopy

TEER is a highly sensitive and reliable measurement of the electrical resistance across a cellular monolayer which confirms the integrity of a cell layer. Moreover, TEER is non-destructive and enables the user to monitor living cells throughout various stages of growth and differentiation. However, mono frequency

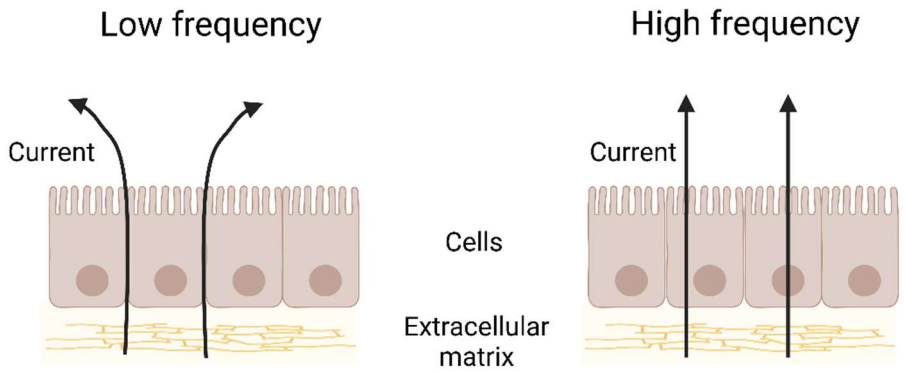

Figure 2. *Current flows at different frequencies. At low frequencies, the primary pathway for current flow is paracellular, indicating the TEER of the cell layer. At high frequencies, the primary pathway is through the cells, indicating the cell capacitance.*

TEER is not able to measure electrical capacitance of the cell layer [19]. Frequency-dependent impedance spectroscopy can measure both TEER and electrical capacitance. At lower frequencies, the primary pathway for current flow is paracellular, moving through extracellular matrix proteins and between adjacent cells via tight junctions, as well as through the electrolyte (medium), see **Figure 2.** Thereby giving insight into the TEER of the cell layer. When high frequencies are applied, the capacitive component of the measured impedance becomes particularly sensitive to adhered cells, see **Figure 2**. At these frequencies, the current travels through the insulating cell monolayer, primarily moving through cell membranes [23]. This highlights the capacitance; cell growth and cell differentiation.

In summary, frequency-dependent impedance spectroscopy enables non-destructive continuous measurement of barrier integrity and cellular development.

A healthy *in vitro* lung model

As discussed before, there is an urgent need for *in vitro* lung models. The characteristics of a healthy lung are an intact cellular barrier with differentiated cells. In our attempt to produce such an *in vitro* healthy lung model, a co-culture was made using primary human bronchial epithelial cell line (NHBE) and primary human lung fibroblasts (NHLF). 1×10^4 NHBE were seeded on the apical side and 1×10^4 NHLF on basal side of a 0.4 µm transwell inserts (Corning) coated with Collagen type I (Sigma-Aldrich, Australia). NHBE cells were maintained in Bronchial epithelial growth medium (BEGM) medium (Lonza, USA) and NHLF cells were maintained in fibroblast growth medium-2 (FGM-2) medium (Lonza, USA). All the cultures were maintained in medium for seven days, then switched to an air-liquid interface (ALI) condition using PneumaCult™-ALI medium (STEMCELL Technolo-gies™, Australia) for a total of 24 days. This co-culture

was chosen because fibroblasts have the capability to enhance epithelial cell function by stimulating proliferation and differentiation, regulating mucin secretion, and initiating a proper spatial distribution.

To examine the electrical resistance and capacitance of cell layers, impedance measurement was performed every two days using our high-throughput automated monitoring system (Locsense, Netherland). Both the apical and basolateral compartments were filled with fresh media (250 µL of medium for apical compartment and 750 µL of medium for basolateral compartment). The impedance of cell layers was measured in a broad range of frequencies (from 10 to 100,000 Hz).

Impedance spectroscopy results revealed an increased TEER when the cell layer formed a barrier, as can be seen in **Figure 3**, Clearly visible is the significant increase of TEER of "Cells on Day 3" compared to TEER "Without cells". The TEER further steadily increases until day 8. After switching to ALI conditions, the TEER slightly decreases until day 15, possibly due to cell differentiation. The cell capacitance is visualized between day 13 to 24 in the right graphs of **Figure 3**. These results show a time dependent increase of cell capacitance from day 13 to day 24. Suggesting that this set-up promotes growth and differentiation of the cells.

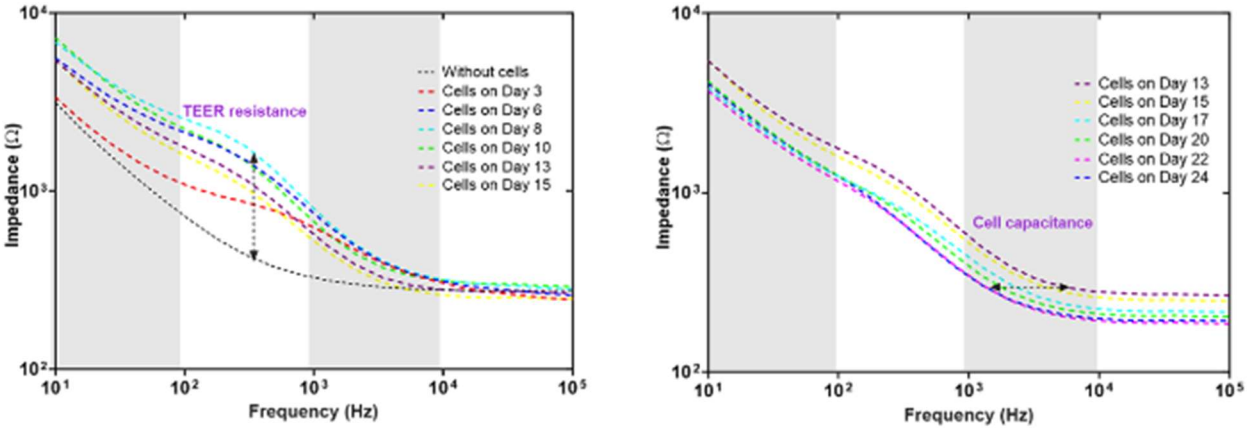

Figure 3. *Impedance measurement of healthy NHBE-NHLF models, performed every 2–3 days for 24 days. The left panels indicated the TEER value from Day 3 to Day 15 and the right panels indicated cell capacitance from Day 13 to Day 24.*

In summary, we developed a model that was subjected to an extensive QC of continuous barrier function and cell differentiation measurements and can be utilized to test safety, efficacy, and superiority of new therapeutics as well as to test toxicity and injury induced by inhaled pollution or pathogens.

References

[1] J. B. Soriano *et al.*, "Prevalence and attributable health burden of chronic respiratory diseases, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017," *Lancet Respir Med*, vol. 8, no. 6, p. 585, Jun. 2020, doi: 10.1016/S2213-2600(20)30105-3.

[2] L. Ruvuna and A. Sood, "Epidemiology of Chronic Obstructive Pulmonary Disease," *Clin Chest Med*, vol. 41, no. 3, pp. 315–327, Sep. 2020, doi: 10.1016/J.CCM.2020.05.002.

[3] S. Kharbanda and R. Anand, “Health-related quality of life in patients with chronic obstructive pulmonary disease: A hospital-based study,” *Indian J Med Res*, vol. 153, no. 4, p. 459, Apr. 2021, doi: 10.4103/IJMR.IJMR_1812_18.

[4] Y. Fu, E. J. Chapman, A. C. Boland, and M. I. Bennett, “Evidence-based management approaches for patients with severe chronic obstructive pulmonary disease (COPD): A practice review,” *Palliat Med*, vol. 36, no. 5, pp. 770–782, May 2022, doi: 10.1177/02692163221079697.

[5] S. G. Bollmeier and A. P. Hartmann, “Management of chronic obstructive pulmonary disease: A review focusing on exacerbations,” *American Journal of Health-System Pharmacy: AJHP*, vol. 77, no. 4, p. 259, Feb. 2020, doi: 10.1093/AJHP/ZXZ306.

[6] A. Cidem, P. Bradbury, D. Traini, and H. X. Ong, “Modifying and Integrating in vitro and ex vivo Respiratory Models for Inhalation Drug Screening,” *Front Bioeng Biotechnol*, vol. 8, p. 581995, Oct. 2020, doi: 10.3389/FBIOE.2020.581995.

[7] G. A. Van Norman, “Limitations of Animal Studies for Predicting Toxicity in Clinical Trials: Is it Time to Rethink Our Current Approach?,” *JACC Basic Transl Sci*, vol. 4, no. 7, pp. 845–854, Nov. 2019, doi: 10.1016/J.JACBTS.2019.10.008.

[8] H. Spits and J. Villaudy, “Modeling human lung infections in mice,” *Nature Biotechnology 2019 37:10*, vol. 37, no. 10, pp. 1129–1130, Sep. 2019, doi: 10.1038/s41587-019-0269-x.

[9] J. S. Tepper, P. J. Kuehl, S. Cracknell, K. J. Nikula, L. Pei, and J. D. Blanchard, “Symposium Summary: ‘breathe In, Breathe Out, Its Easy: What You Need to Know about Developing Inhaled Drugs,’” *Int J Toxicol*, vol. 35, no. 4, pp. 376–392, Jul. 2016, doi: 10.1177/1091581815624080/ASSET/IMAGES/LARGE/10.1177_1091581815624080-FIG7.JPEG.

[10] F. Yu, W. Hunziker, and D. Choudhury, “Engineering Microfluidic Organoid-on-a-Chip Platforms,” *Micromachines 2019, Vol. 10, Page 165*, vol. 10, no. 3, p. 165, Feb. 2019, doi: 10.3390/MI10030165.

[11] K. Brune, J. Frank, A. Schwingshackl, J. Finigan, and V. K. Sidhaye, “Pulmonary epithelial barrier function: some new players and mechanisms,” *Am J Physiol Lung Cell Mol Physiol*, vol. 308, no. 8, p. L731, Apr. 2015, doi: 10.1152/AJPLUNG.00309.2014.

[12] K. Brown and D. T. Lynch, “Histology, Lung,” *StatPearls*, May 2023, Accessed: Dec. 05, 2023. [Online]. Available: https://www.ncbi.nlm.nih.gov/books/NBK534789/

[13] P. J. Barnes, “Club cells, their secretory protein, and COPD,” *Chest*, vol. 147, no. 6, pp. 1447–1448, Jun. 2015, doi: 10.1378/chest.14-3171.

[14] A. Herminghaus *et al.*, “A Barrier to Defend - Models of Pulmonary Barrier to Study Acute Inflammatory Diseases,” *Front Immunol*, vol. 13, p. 1, Jul. 2022, doi: 10.3389/FIMMU.2022.895100.

[15] J. A. Frank, “Claudins and alveolar epithelial barrier function in the lung,” *Ann N Y Acad Sci*, vol. 1257, no. 1, p. 175, 2012, doi: 10.1111/J.1749-6632.2012.06533.X.

[16] F. M. Carlier, C. de Fays, and C. Pilette, “Epithelial Barrier Dysfunction in Chronic Respiratory Diseases,” *Front Physiol*, vol. 12, p. 691227, Jun. 2021, doi: 10.3389/FPHYS.2021.691227/BIBTEX.

[17] N. Rout-Pitt, N. Farrow, D. Parsons, and M. Donnelley, “Epithelial mesenchymal transition (EMT): a universal process in lung diseases with implications for cystic fibrosis pathophysiology,” *Respiratory Research 2018 19:1*, vol. 19, no. 1, pp. 1–10, Jul. 2018, doi: 10.1186/S12931-018-0834-8.

[18] D. Bartis, N. Mise, R. Y. Mahida, O. Eickelberg, and D. R. Thickett, “Epithelial–mesenchymal transition in lung development and disease: does it exist and is it important?,” *Thorax*, vol. 69, no. 8, pp. 760–765, Aug. 2014, doi: 10.1136/THORAXJNL-2013-204608.

[19] B. Srinivasan, A. R. Kolli, M. B. Esch, H. E. Abaci, M. L. Shuler, and J. J. Hickman, “TEER measurement techniques for in vitro barrier model systems,” *J Lab Autom*, vol. 20, no. 2, p. 107, Apr. 2015, doi: 10.1177/2211068214561025.

[20] N. J. Severs, “Freeze-fracture electron microscopy,” *Nat Protoc*, vol. 2, no. 3, pp. 547–576, Mar. 2007, doi: 10.1038/NPROT.2007.55.

[21] C. M. Van Itallie and J. M. Anderson, “Architecture of tight junctions and principles of molecular composition,” *Semin Cell Dev Biol*, vol. 0, p. 157, Dec. 2014, doi: 10.1016/J.SEMCDB.2014.08.011.

[22] H. Kadry, B. Noorani, and L. Cucullo, “A blood–brain barrier overview on structure, function, impairment, and biomarkers of integrity,” *Fluids and Barriers of the CNS 2020 17:1*, vol. 17, no. 1, pp. 1–24, Nov. 2020, doi: 10.1186/S12987-020-00230-3.

[23] K. Benson, S. Cramer, and H. J. Galla, “Impedance-based cell monitoring: Barrier properties and beyond,” *Fluids Barriers CNS*, vol. 10, no. 1, pp. 1–11, Jan. 2013, doi: 10.1186/2045-8118-10-5/FIGURES/14.