# A Powerful Tool for the Study of Airway Biology: Human Tracheobronchial Epithelial Model Grown at the Air-liquid Interface

Application Note

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## Introduction

Normal human tracheobronchial epithelium protects the airways by providing a barrier against injury from external insults. Intact cellular organization and function of the epithelial barrier provides a sterile and stable inner environment for living organisms. Ciliated and nonciliated columnar cells, secretory cells, and basal cells are arranged in a pseudostratified pattern to form complex epithelium structure and function. Two distinct compartments, apical and basolateral are developed by all cells creating tight junctions and contacting basement membrane, while some cells will reach the luminal surface[1]. When exposed to particles such as pollutants, bacteria, or virus, these particles will be cleared by the co-operation of ciliated cells and secretory cells[2].

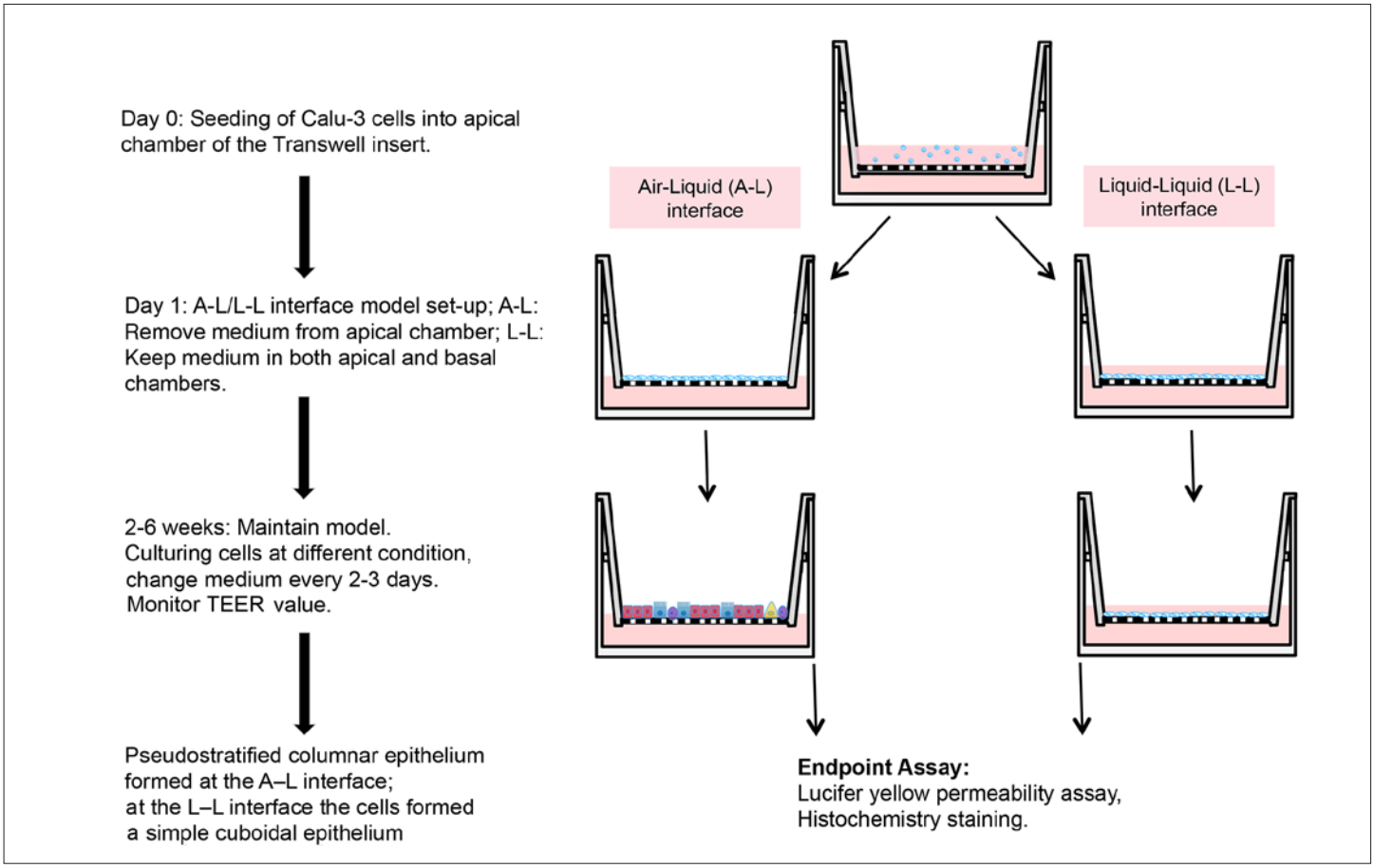
Human tracheobronchial epithelial cells grown at the air-liquid interface (ALI) culture have emerged as a powerful tool for the study of airway biology. Air-liquid interface culture for 3 to 6 weeks can result in the formation of well-differentiated, polarized human tracheobronchial epithelial cultures that resemble *in vivo* pseudostratified mucociliary epithelium. *In vitro* models using primary cells and cell lines are essential for understanding the function of the epithelium. Studies with cell lines are convenient, reproducible, and inexpensive compared to experiments involving primary cells. Calu-3, an epithelial cell line derived from human lung adenocarcinoma, has been widely used in studying bronchial epithelium, its closest resemblance to this epithelium *in vivo*[3]. ALI models of culturing epithelial cells has shown its application in response to stimulations including wounding, anion secretion, and viral infection[4,5,6].

The Transwell® permeable support insert from Corning Life Sciences is a useful tool that mimics an *in vivo* environment, which is why it has been widely used in a variety of studies, including drug transport/absorption, cell migration and differentiation, and co-culture applications. In addition, several *in vitro* models have also been constructed to better explore the natural physiology of cellular mechanism.

## Reagents and Materials

- Calu-3 cell line (ATCC Cat. No. HTB-55)
- MEM medium (Corning Cat. No. 10-010-CV)
- Fetal bovine serum (FBS, Corning Cat. No. 35-076-CV)
- Transwell permeable support insert (Corning Cat. No. 3470, 0.4 µm PET membrane)
- 24-well plate (Corning Cat. No. 3524)
- 96-well assay plate (Corning Cat. No. 3915)
- Lucifer yellow (Sigma Cat. No. L0144)
- HBSS (Corning Cat. No. 21-020-CV)
- HEPES (Corning Cat. No. 25-060-Cl)
- Voltohmmeter (MilliporeSigma)
- SpectraMax® M4 plate reader (Molecular Devices)

## Workflow




## Procedure

### 1. Model Generation

Cells were seeded into Transwell® inserts on day 0. Prior to seeding, Transwell inserts were pre-incubated at 37°C with culture medium for at least 30 minutes. Calu-3 cells were seeded into the apical chamber of the Transwell inserts at densities of 0.25 x $10^5$, 0.5 x $10^5$, 1 x $10^5$, 2 x $10^5$, and 4 x $10^5$ cells/well with 150 µL of culture medium. 800 µL of medium was added to the basal chamber. The plate was placed in a humidified 37°C/5% $CO_2$ incubator and cultured overnight. On day 1 for the A-L interface, the culture medium in apical chamber was removed after seeding for 24 hours, and cells were cultured with 500 µL culture medium in the basal chamber. For L-L interface, the medium in both apical and basal chambers were kept. Culture medium was changed every 2 to 3 days, and cultured for 3 weeks.

### 2. TEER Value Monitor

The TEER value was measured every 2 to 3 days using a Voltohmmeter (MilliporeSigma) to confirm the development of tight junctions. Both the basolateral and apical chamber were filled with fresh pre-equilibrated medium and TEER was read after allowing the cell culture to get stable potential for approximately 15 to 30 min. to avoid any temperature fluctuation-induced TEER changes, because TEER measurement is sensitive to temperature[7]. Medium was removed from the apical side of the insert immediately after TEER readings to re-establish the ALI condition.

### 3. Lucifer Yellow Permeability Assay

On day 21, the Lucifer yellow permeability assay was performed. To prepare the donor solution, 100 mM stock solution of Lucifer yellow was diluted with HBSS/10 mM HEPES buffer to 300 µM. The receiver solution used was HBSS/HEPES buffer with a DMSO content matched to that in the donor solution. The Transwell inserts were washed once with HBSS/HEPES buffer, transferred into a new 24-well receiver plate with receiver solution, and then 50 µL of the donor solution was added to the apical chamber. The plate was placed at 37°C with orbital shaking for 60 min. Then 100 µL of the solution in the receiver plate was transferred to a black assay plate and the fluorescence was read at 480 nm/530 nm using a SpectraMax® M4 plate reader. In the meantime, a series of Lucifer yellow solution with different concentrations were diluted and the standard curve was plotted. The concentration of Lucifer yellow in the receiver plate was calculated from the standard curve.

### 4. Cell Staining

After culturing in the Transwell® inserts, Calu-3 cells, together with the Transwell membrane, were cut from the insert and fixed in 10% formalin overnight at 4°C. After dehydration and paraffin embedment, 5 µm sections were prepared and stained with hematoxylin and eosin (H&E), periodic acid–Schiff (PAS) stain for neutral proteoglycans, or Alcian blue for acid proteoglycans, and viewed with a lighted microscope.

To demonstrate mucus cell differentiation, confluent layers of cells grown at ALI model were subjected to PAS staining to visualize neutral and acidic glycoproteins (likely mucins).

### 5. Calculations

**Transepithelial Electrical Resistance (TEER):**

TEER of insert and medium alone was subtracted from measured TEER and calculated multiplying by the insert area.

$$TEER = (R_m - R_b) \times A$$

Where:

TEER is the transepithelial electrical resistance

$R_m$ is the resistance reading obtained for the cell monolayer

$R_b$ is the resistance reading obtained for the blank insert (without cells)

A is the surface area of insert filter membrane [$cm^2$]

**Apparent Permeability ($P_{app}$):**

$$P_{app}[cm/s] = \frac{Flux \times V_d}{t \times A} = \frac{dQ}{dt} \times \frac{1}{A \cdot C_0}$$

Where:

Flux is the fraction of the donated amount recovered in the receiver chamber

$V_d$ is the volume in the donor chamber [$cm^3$]

$C_0$ is the initial concentration in the donor solution [mM]

A is the surface area of insert filter membrane [$cm^2$]

t is the incubation time [sec.]

dQ/dt is the amount of drug transported within a given time period [pmol/sec.]

## Results

### 1. Microscopic Observation

In the L-L interface model, Calu-3 cells became confluent and lacked clear structure after 3 weeks of culturing in the Transwell inserts. In the A-L model, protuberances were observed after 3 weeks cultivation (Figure 1).

### 2. Evaluation of Cell Monolayer Integrity

Transepithelial electrical resistance (TEER) is a widely accepted quantitative technique to measure the integrity of tight junction dynamics in cell culture models of epithelial monolayers. TEER

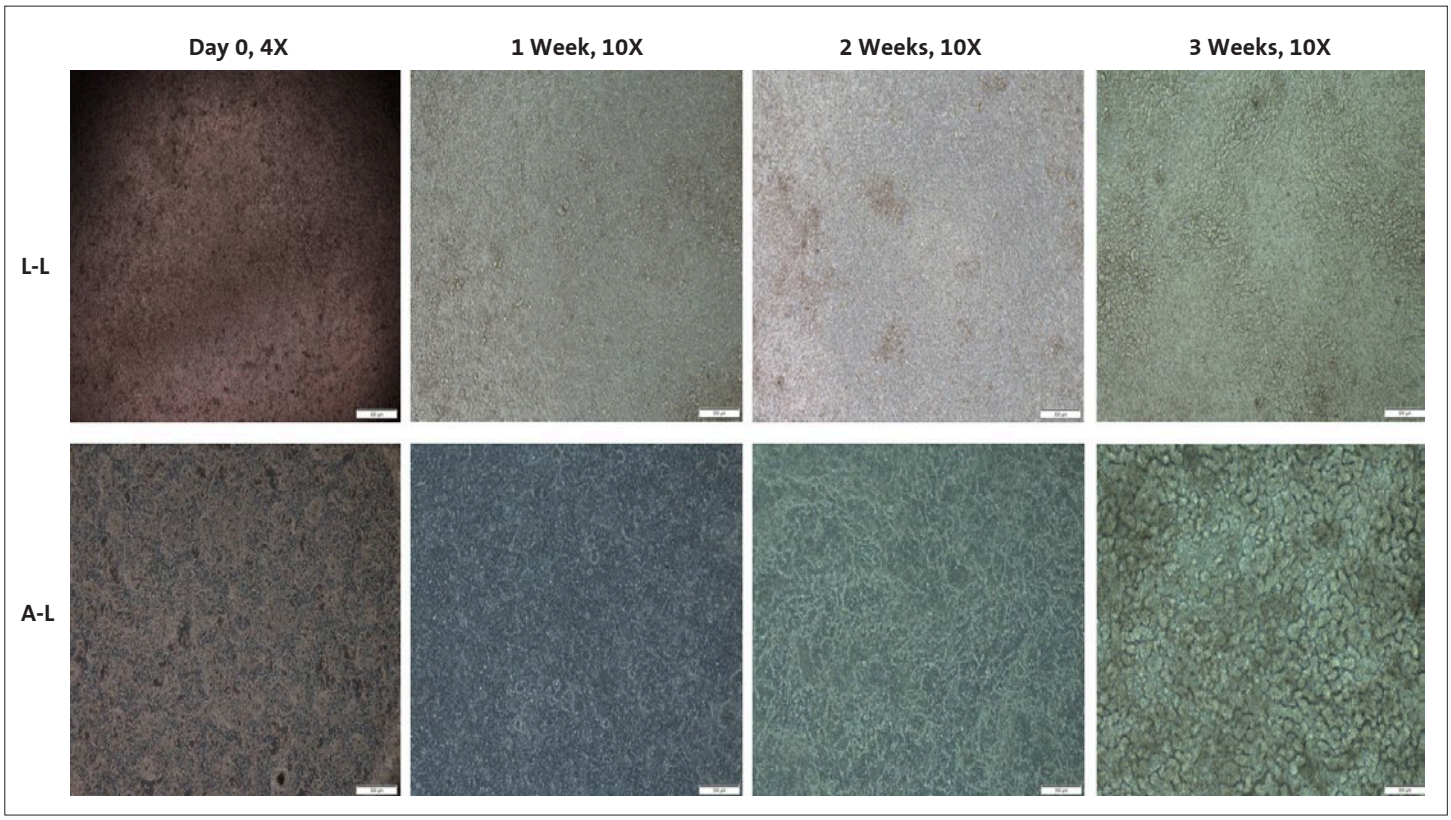



**Figure 1. Calu-3 bronchial epithelial model.** Cultivation of Calu-3 cell at L-L model (upper) and A-L model (below). Microscopy images photos were taken at day 0, 1 week, 2 weeks, and 3 weeks under microscope.

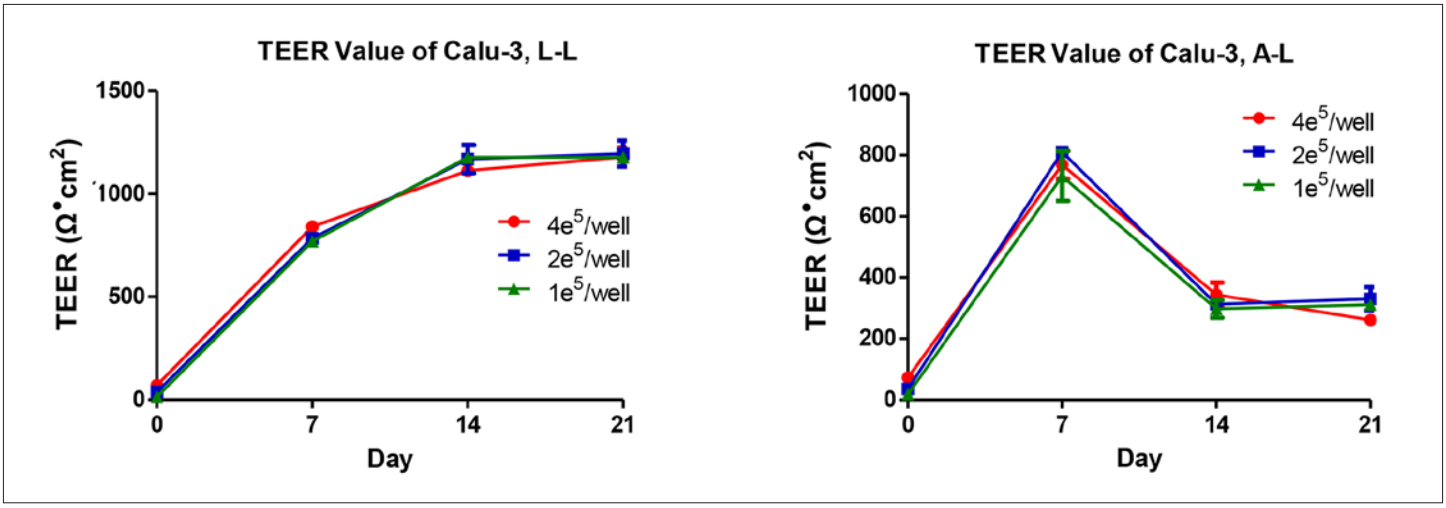



**Figure 2. TEER measurements of Calu-3 in L-L and A-L models.** Time-course of TEER development for the L-L model (left) and A-L model (right) at day 7, 14, and 21. Seeding densities of Calu-3 cells: 4e5 (red squares), 2e5 (blue dots), and 1e5 (green triangles) per insert.

values are strong indicators of the integrity of the cellular barriers before they are evaluated for transport of drugs or chemicals. TEER measurements can be performed in real-time without cell damage and generally are based on measuring ohmic resistance or measuring impedance across a wide spectrum of frequencies.

In the L-L interface model, the TEER values of Calu-3 cells reached more than 1,000 Ω•cm$^2$ at day 14 and remained stable until day 21. In the A-L interface model, high TEER values were reached in one week, then decreased and stabilized at approximately 300 Ω•cm$^2$ (Figure 2).

Lucifer yellow was also used as a paracellular marker for monolayer integrity to confirm the results of TEER measurements. Lucifer yellow travels across the cell monolayer only through passive paracellular diffusion and has low permeability. It is not able to pass across cell monolayers when tight junctions between cells are maintained. Figure 3 shows the permeability ($P_{app}$) of Lucifer yellow for Calu-3 epithelial L-L and A-L models. $P_{app}$ was much lower than 1 x 10$^{-6}$ cm/s for both L-L and A-L models. Tight junctions were formed after 3 weeks of cultivation in the A-L model.

### 3. Haematoxylin-eosin (HE) Staining

The results of HE staining indicated that pseudostratified columnar epithelium was formed in 3 weeks in the A-L model (Figure 4).

Cells exhibited poor morphological quality in the L-L model. They appeared dedifferentiated, flat, or dwarf, with reduced numbers of cilia and appearance of secretory protrusions in the L-L model; While in the A-L model, cells were highly polarized, confluent, and retained a mix of ciliated and secretory cells.

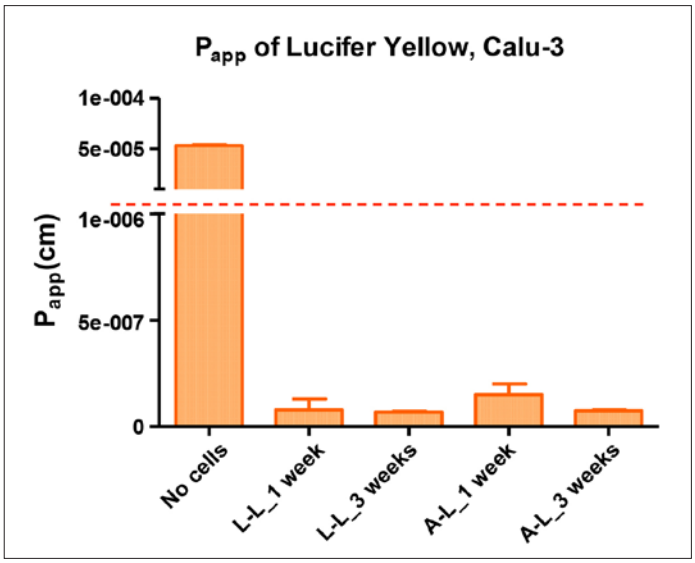



**Figure 3. Lucifer permeability of the Calu-3 epithelial models after 1 week and 3 weeks.**

### 4. Periodic Acid Schiff (PAS) and Alcian Blue Staining of Proteoglycans/Mucus

Neutral proteoglycans were stained with PAS (red) and nuclei were stained with nuclear fast red (Figure 5, left panel). Acid proteoglycans were stained with Alcian blue (blue; Figure 5, right panel). The results showed that secretory products and vesicles were visible in cells cultured at the A–L interface for 3 weeks.

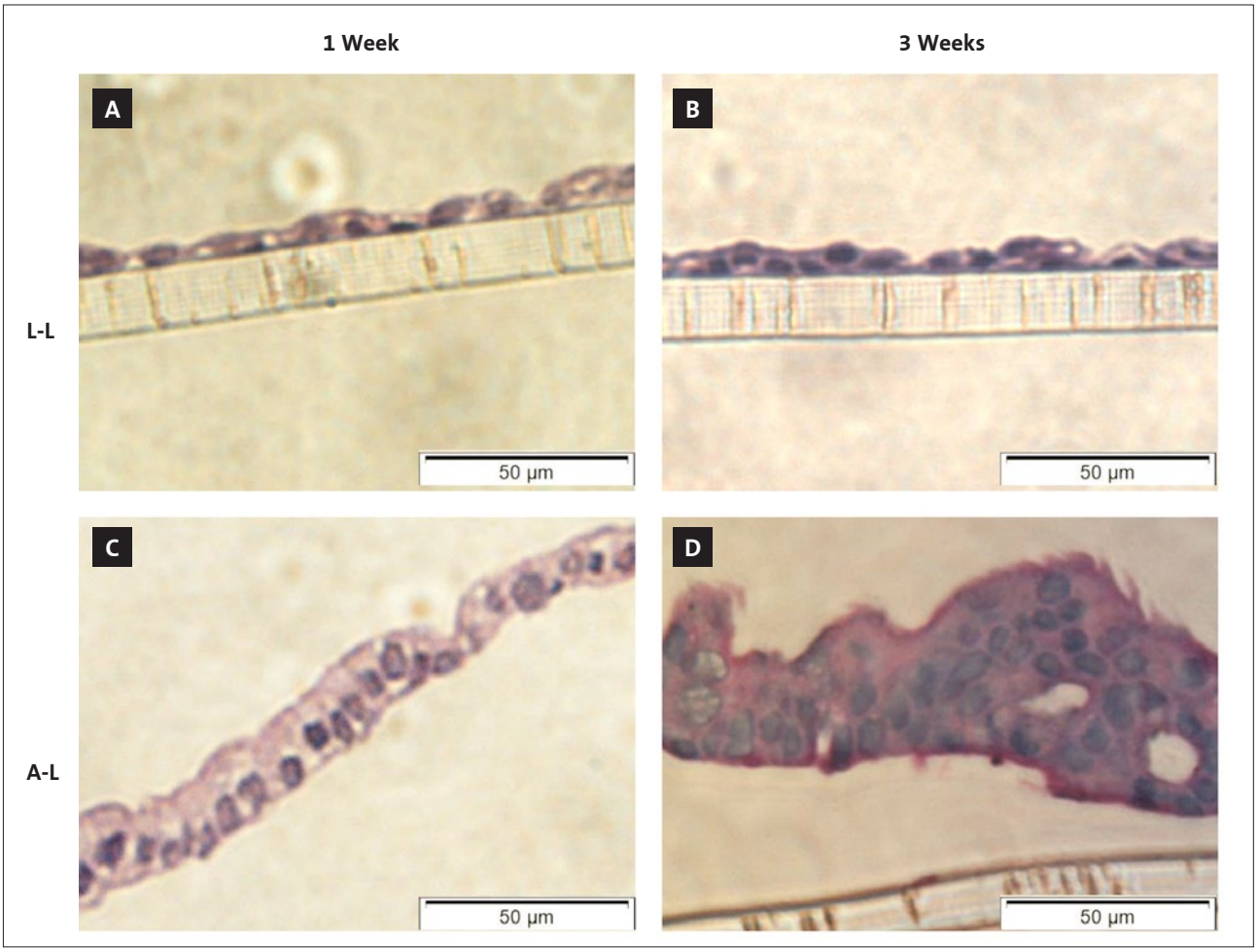



**Figure 4. Haematoxylin-eosin stain of Calu-3 cells at the L-L and A-L interfaces after 1 week and 3 weeks in culture.** Haematoxylin-eosin stain at the L-L interface revealed a flat cell monolayer (A, B). At the A-L interface, pseudostratified mucociliary epithelium morphology was formed (C, D).

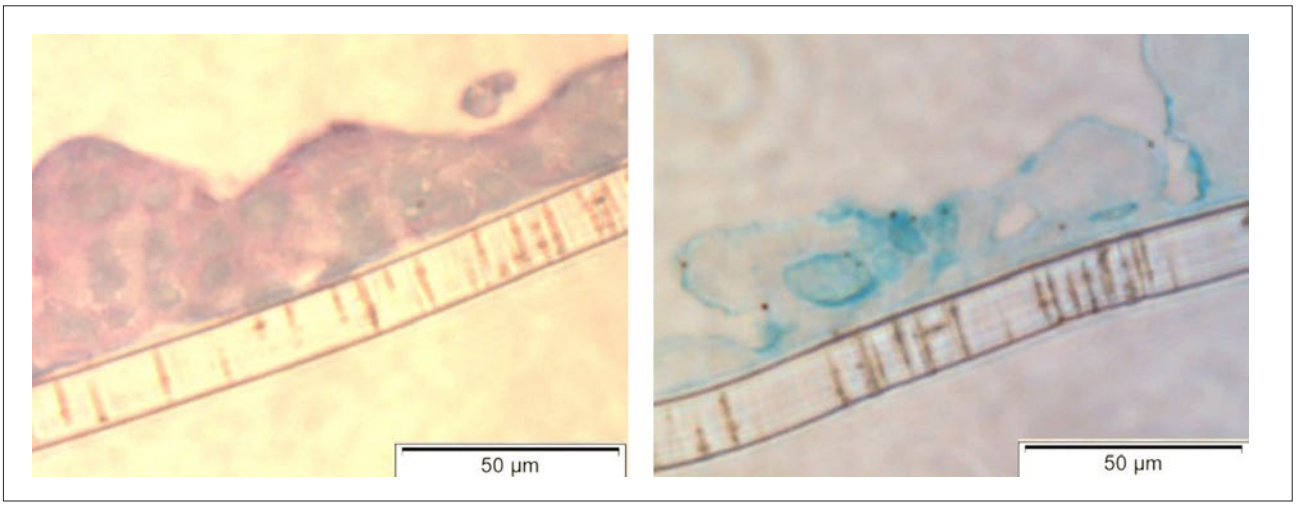



**Figure 5. PAS (left) and Alcian blue stain (right) for Calu-3 cells at the A-L interfaces at 3 weeks in culture.**

## Conclusion

The study of airway biology can be achieved by cultivation of human tracheobronchial epithelial cells, primary cells, and cell lines at the air-liquid interface. In the present study, a successful bronchial epithelial model had been set up by using Calu-3 cells cultivated at the air-liquid interface for 3 weeks. Several methods were performed to verify the integrity of this model, including measurement of TEER value for tight junctions between cell monolayers and Lucifer yellow permeability. TEER reflects the ionic conductance of the paracellular pathway in the epithelial monolayer. This method is non-invasive and can be applied to monitor liver cells during their various stages of growth and differentiation. However, various factors have been shown to affect TEER values, including temperature, medium formulation, passage number of cells, and usage of electrodes[7]. Histochemical staining confirmed the pseudostratified mucociliary epithelium morphology.

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