Modeling a 3D respiratory model with the Quasi Vivo® QV600 Fluidic Cell Culture System
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Introduction

In order to study pathomechanisms in human disease, relevant model systems are required that mimic the *in vivo* conditions in the human patient. Most *in vitro* studies of the human lung make use of monolayer-based two-dimensional (2D) cultures grown on non-permeable plastic or glass surfaces, which prevent the cells from establishing a three-dimensional (3D) architecture, critical for their differentiation to mimic the structure and function of parental tissues *in vivo*. An additional downside to 2D monolayer cultures is the inability to react to various chemical and molecular gradients due to lack of apical, basal and lateral cell structures.[1,2] Current approaches to recapitulate human lung diseases *in vitro* involve the use of normal human primary epithelial cells of nasal, bronchial or tracheal origin, which are typically cultured on bio-compatible scaffolds or matrices (collagen, alginate, gelatin, elastin, basement membrane extract) to mimic the *in vivo* environment. As a further step towards improving the physiologic relevance of these models, these primary cells are cultured in Transwell inserts in an Air-Liquid – Interface (ALI) culture that results into differentiation of these cells within a defined spatial orientation to produce basal cells, ciliated cells and mucus-producing goblet cells, thus mimicking conditions *in vivo.*[3]

While standard ALI culture results in a more *in vivo* like cell architecture, one other key component of a physiological cell environment is lacking. Most cell types are exposed to continuous fluid flow, for example blood or interstitial fluid flow, in the human body. In order to mimic this, we used the commercially available ALI perfusion chamber bioreactor (Quasi Vivo® QV600 System, Lonza) to keep the cells in ALI culture under continuous perfusion from the basolateral side (Figure 1). The main feature of this system is the ability to apply various flow rates dependent on the cell type and providing constant nutrient turnover to cells without imposing high shear stress or turbulent flow.

In this Technical Note, we provide detailed information of how the Quasi Vivo® QV600 System was set up for the ALI culture of normal human bronchial or small airway epithelial (NHBE, SAE) cells. We show that the perfusion culture resulted in significantly accelerated development of the lung epithelia associated with higher ciliogenesis, cilia movement, mucus-production and improved barrier function compared to growth under static conditions. This faster differentiation of 3D respiratory models allows an earlier start of the "real" applications within the system such as cytokine measurements following exposure, characterization of treated versus untreated cells, or viability assays to name a few.

Materials and methods

Cell culture

Primary normal human bronchial epithelial (NHBE) or small airway (SAE) cells were obtained from Lonza (cat. no.: CC-2540S and CC-2547S). The cells were cultured in a T75 flask for 2 – 4 days until they reached 80% confluence. The cells were trypsinized and seeded onto collagen (Corning collagen I, rat tail)-coated 0.33 cm^2 porous (0.4µm) polyester membrane inserts with a seeding density of 1 x 10^5 cells per Transwell (Costar, Corning). The cells were grown to near confluence in submerged culture for 2 – 3 days in specific epithelial cell growth medium (Lonza, BEGM™ Bronchial Epithelial Medium, cat. no. CC-3170; SAGM™ Small Airway Epithelial Medium, cat. no. CC-3118) according to the manufacturer´s instructions. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C and then transferred to ALI culture, by completely removing the cell culture medium from the apical part of the insert. The epithelium was fed with B-ALI™ or S-ALI™ Differentiation Medium (Lonza, cat. nos.: 193514, CC-4539) from the basal side by adding 500 µL of the culture medium into the cell culture well. The number of days in development was designated relative to initiation of ALI culture, corresponding to day 0. Dependent on the conditions (static vs. perfused) cells were used from day 14 or 21 in ALI for experiments.

Δ Critical step:

Culturing cells for longer periods in ALI resulted – as expected – in increased cilia formation and mucus production. These changes in the ALI cultures over time must be taken into account when planning experiments. For biological replicates and when comparing different culture conditions or treatments, cultures of the same age, i.e. differentiated for the same number of days in ALI cultures, must be used to exclude any undesired bias caused by different ages of the respective cultures. We used cells cultured in ALI for 30 to 70 days for most experiments.

Set up of the dynamic perfused cell culture system

A commercially available ALI perfusion chamber bioreactor (Quasi Vivo® QV600 Dual Flow Starter Kit, Lonza, cat. no. QVTWSK) was used to keep the cells under perfusion (Figure 1). The main feature of this system is the ability to apply various flow rates dependent on the cell type and providing constant nutrient turnover to cells without imposing high shear stress or turbulent flow.[4] After pre-culturing NHBE or SAE cells on Transwells under submerged conditions as described above, inserts were lowered inside the chamber base from above and allowed to rest and hang from the inner lip of the base in the QV600 Chamber (ALI). The medium from the apical side of the insert was removed to create an ALI culture. The lid of the QV600 Chamber was fastened to seal in and immobilize the insert. Quasi Vivo® QV600 Chambers and a mixing chamber (ALI medium reservoir) were then connected in series to a peristaltic pump (Ismatec, IPC ISM756) giving a final volume of cell culture media of about 25 mL (Figure 2).

Δ Critical step:

A dual pump connection was used for active gas exchange. The schematic (Figure 2A) represents the set-up used to culture the lung epithelial cells. A single air filter was attached to the first chamber. The second chamber was kept closed by connecting the tubing entering and leaving the upper

part of the QV600 Chamber. A dual pump connection across both chamber input and output lines using a single Ismatec peristaltic pump was established. This combination of one single air filter in the first chamber with the dual pump connection was required to maintain a constant medium level in the cell culture chambers while actively drawing air into the chamber via the filter connected to the first chamber. Alternative set-ups could result in flooding or too low medium levels in the QV600 Chambers.

For maintaining a stable liquid flow rate in the chosen dual pump-connection set-up, the flowrate at P1 should be less than the flowrate at P2 i.e., P1< P2 (Figure 2B). The difference in flow rate was obtained by using tubing with different diameters. A tubing with an inner diameter of 1.6 mm was used to transport medium into the lower part of the QV600 Chamber. The tubing with an inner diameter of 2.4 mm was connected to the QV600 Media Outlet. An empirical approach was used here by trying different tubing diameters and the best tubing dimensions as mentioned above were used in our set up. However depending on the requirements of different users, the tube dimensions could vary depending on the type of culture and cells used.

Figure 1

Quasi Vivo® Interconnected System (op), QV600 ALI (bottom) (Lonza, cat. no. QVTWSK). Quasi Vivo® QV600 Silicone Chambers in which cell culture inserts are introduced and allowed to hang from the inner lip of the base of the chamber, the lid is fastened to seal in and immobilize the insert while culturing the respiratory epithelial cells.

The medium was pumped through the chambers at a rate of 150 µL/min maintaining an air-liquid interphase. The medium in the reservoir bottle was re-fed every week with 10 – 15 mL of the specific medium to maintain a final volume of 25 mL.

Δ Critical step:

A marking was made on the reservoir bottle to indicate the liquid medium. This aided monitoring any alterations in the level and amount of liquid used inside the system.

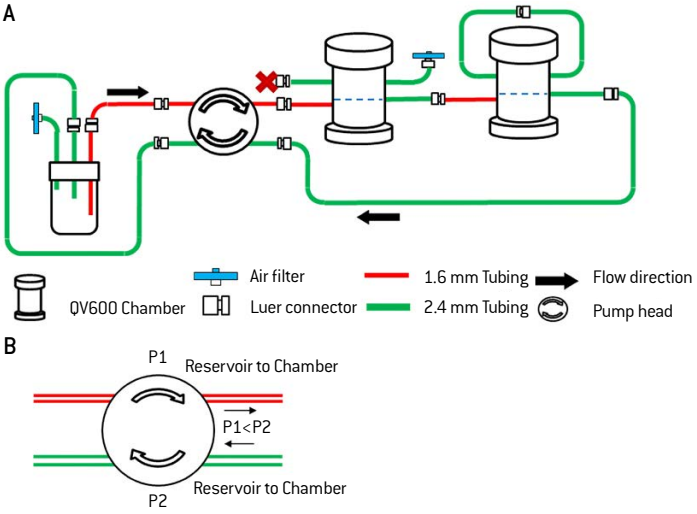


Figure 2

Schematic of the set-up of the Dynamic Perfusion System: (A) Two QV600 Chambers are connected in series to an ISMATEC Pump with one air filter between the two chambers. (B) Dual connection to the same ISMATEC pump maintaining a consistent liquid level with flow rate at P1< P2.

Scanning electron microscopy (SEM)

For SEM analyses, cultures were fixed with 2.5% glutaraldehyde EM Grade R1012 (BioChemika Fluka) in 0.1 M phosphate buffer (pH 7.4) solution overnight. The next day, samples were washed with sterile filtered D-PBS solution and gradually dehydrated using ethanol. Sputter coating was performed with gold and a Jeol6010 in Touch (Jeol Germany) was used for analyses. Samples were examined with a field emission scanning electron microscope (Gemini 982, Zeiss).

TEER measurement

TEER values were measured using EVOM voltohmmeter with STX-2 chopstick electrodes (World Precision Instruments, Stevenage, UK).

Δ Critical step:

In order to disturb ALI cultures in their respective plates or culture chambers as little as possible, TEER measurements of ALI cultures were aligned with the regular media changes. ALI cultures were used for TEER measurements immediately before a medium was necessary. After TEER measurements were completed, the ALI cultures were put back into their original cell culture system containing fresh culture medium.

For measurements, the pump flow was stopped, chambers were opened under sterile conditions and the Transwells transferred to a sterile 24- well plate. 0.2 mL and 1.0 mL of medium were added to the apical side and basolateral sides, respectively. Cells were allowed to equilibrate for 5 mins before TEER was measured. TEER values reported were corrected for the resistance and surface area of the Transwell filters. This was the standard method employed for measuring TEER for cells cultured under static or perfused conditions.

Results and discussion

Superior performance and accelerated development of airway epithelial cells using dynamic perfused culture conditions

Culturing the normal human bronchial epithelial cells under perfused conditions using the Quasi Vivo® QV600 Chambers resulted in faster development of the epithelial cells in that the cells exhibited highly developed tight junctions (red, Occludin) and high mucus production (lilac, MUC5B) after 7 days in ALI (Figure 3). On the contrary, under static conditions at day 7 in ALI, the NHBE cells showed a decreased level of differentiation that was evident from the lack of mucus production and lower expression of tight junctions. While some amount of mucus production was seen after 3 weeks in static conditions, this was not comparable even with mucus production seen at day 7 under perfusion. The tight junction formation of NHBE cells cultured for 3 weeks under static conditions was comparable to the cells cultured for 7 days under perfusion (Figure 3).

Scanning electron microscopic analyses and live cell imaging indicated higher ciliogenesis under perfused conditions compared to static conditions on day 7 in ALI (Figure 4). Under perfusion, higher density of cilia was visible on the surface of the epithelia in comparison with static conditions where ciliogenesis was marginal (Figure 4).

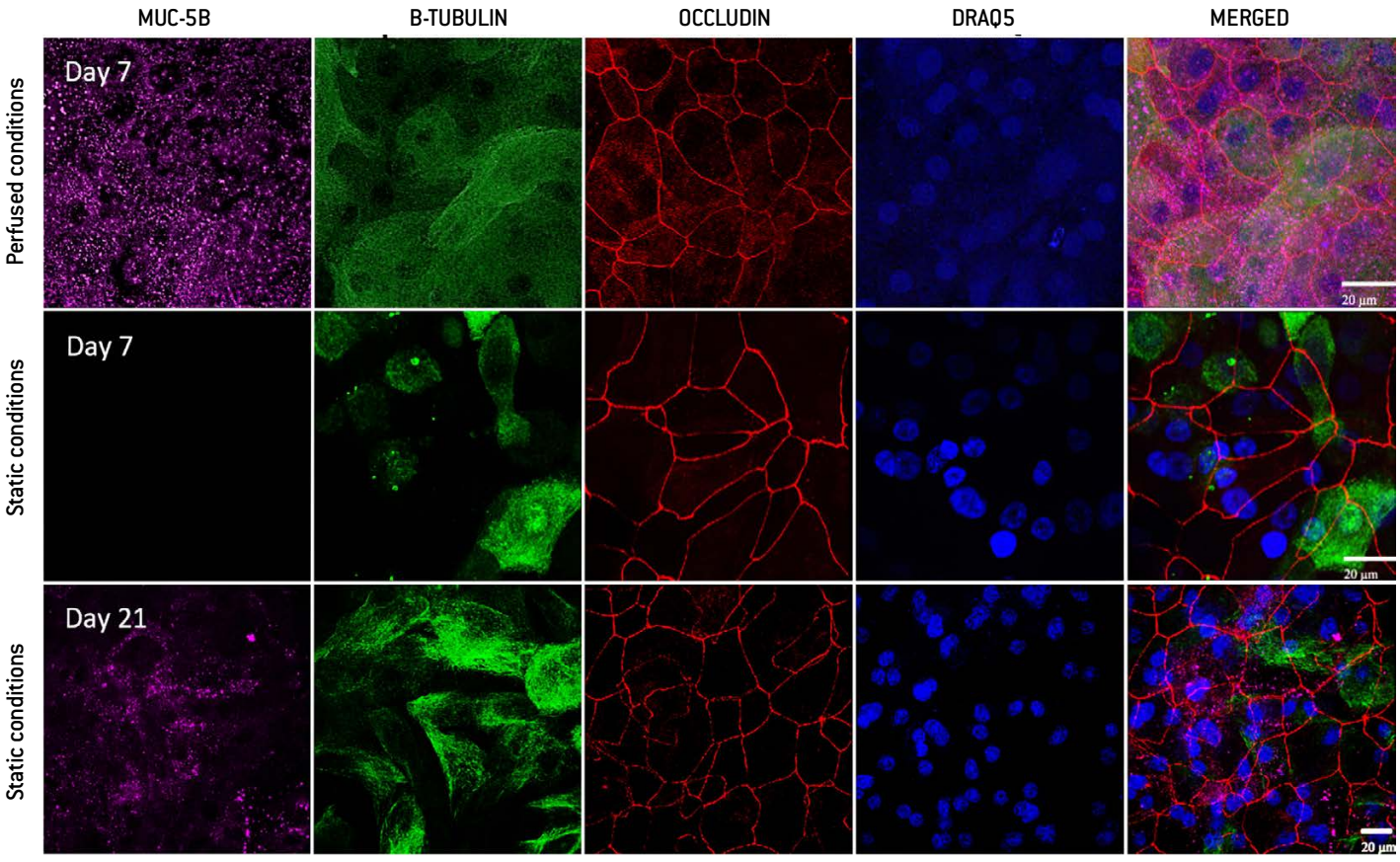


Figure 3

Superior growth of respiratory cells in ALI under perfused conditions. NHBE cells when cultured in a dynamic perfused system (upper panel) were fully differentiated on day 7 in ALI – they illustrated high amounts of mucus production (MUC5B, lilac), cilia staining (tubulin, green) and tight junctions (occluding, red), while under static conditions no mucus was produced at all on day 7 (middle panel). Mucus production under static conditions started around day 21 and at this time also cilia and well differentiated tight junctions were formed (lower panel). Nuclei were stained using Draq5 (blue) and overlays are depicted in the last row (merged).

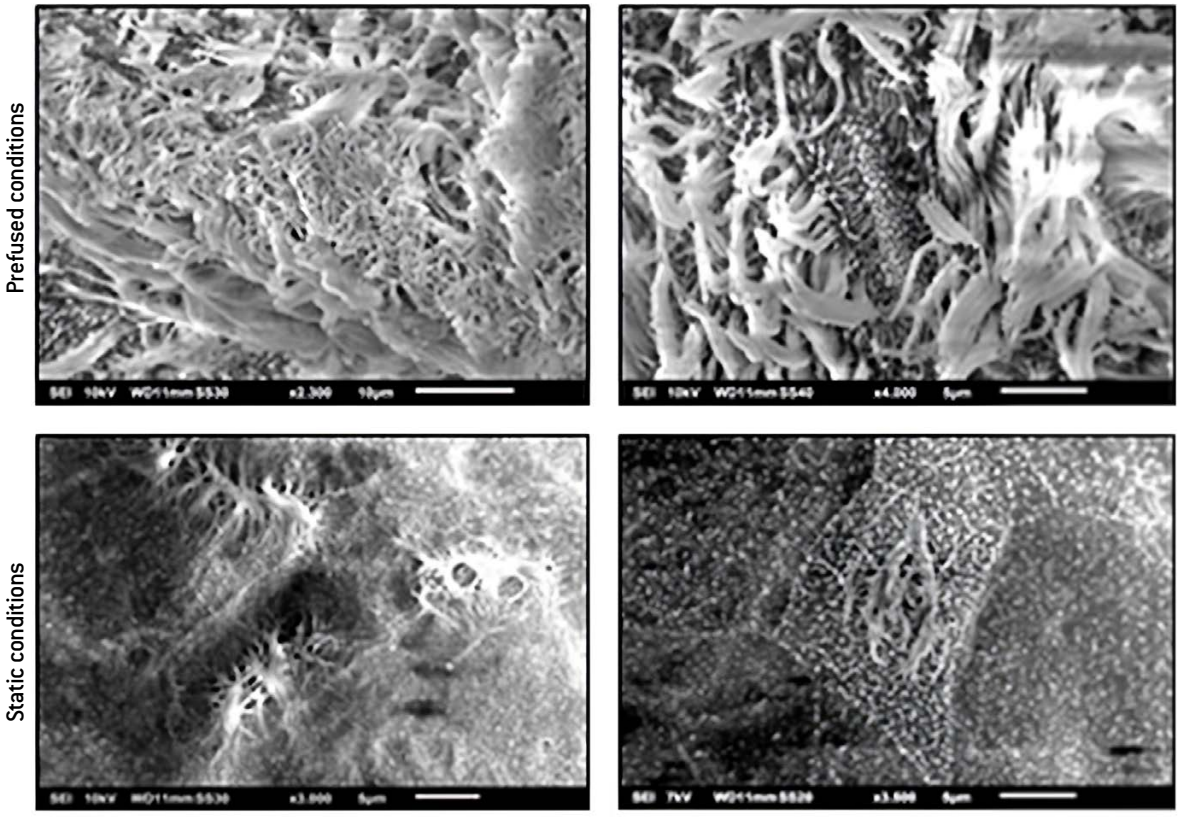


Figure 4
Higher ciliogenesis of NHBE cells cultured under perfused conditions compared to static conditions. The higher differentiation of NHBE cells grown under perfusion (upper panel) compared to static conditions (lower panel) on day 7 post ALI was also illustrated by SEM. SEM analyses were performed with cells from at least three different Transwells.

Similar results were obtained when surface staining live NHBE cells cultured under perfused conditions using fluorescently labelled wheat germ agglutinin (WGA), which binds to glycoproteins of the cell membrane. Figure 5 depicts the high cilia development (WGA, green) of Mitotracker-stained living cells (red) grown under perfusion. Nuclei stained with Hoechst are illustrated in blue (Figure 5).

Similar results were obtained using small airway epithelial (SAE) cells grown under perfused or static conditions. The integrity of SAE cells grown under perfusion is illustrated in Figure 6. We demonstrate here a significantly accelerated development and differentiation of lung epithelial cells independent of origin under perfusion conditions.

Monitoring the barrier function of the respiratory epithelial cells cultured under static and perfused conditions

The functionality of the epithelial barrier was evaluated by monitoring the trans-epithelial electrical resistance (TEER) for the two types of respiratory epithelial cells. TEER, which is a functional parameter to monitor the quality of epithelial cells such as membrane integrity and various stages of growth and differentiation, was measured over time using cells grown under perfusion or static conditions. A continuous

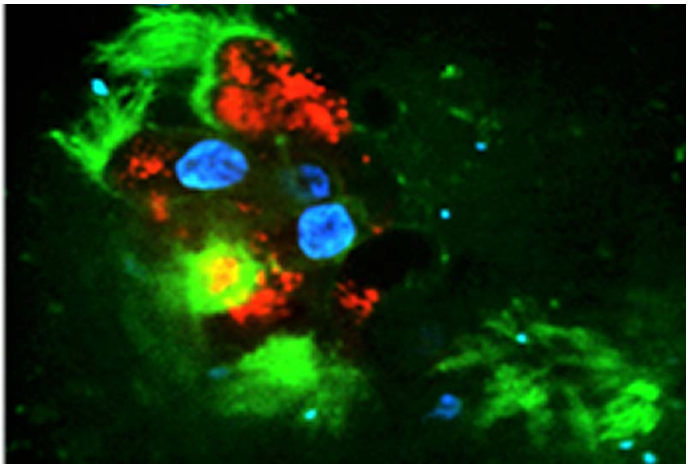


Figure 5
Live cell imaging showing higher ciliogenesis of NHBE cells cultured under perfused conditions. Live cell imaging of cells grown under perfusion also showed the high differentiation grade of these cells. The surface of live epithelial cells grown under perfusion was stained using wheat germ agglutinin (WGA, green), while intracellular staining comprised Mitotracker for mitochondria (red) and Hoechst as nuclear stain (blue). For CLSM at least five different areas each containing ~25 cells per condition were captured.

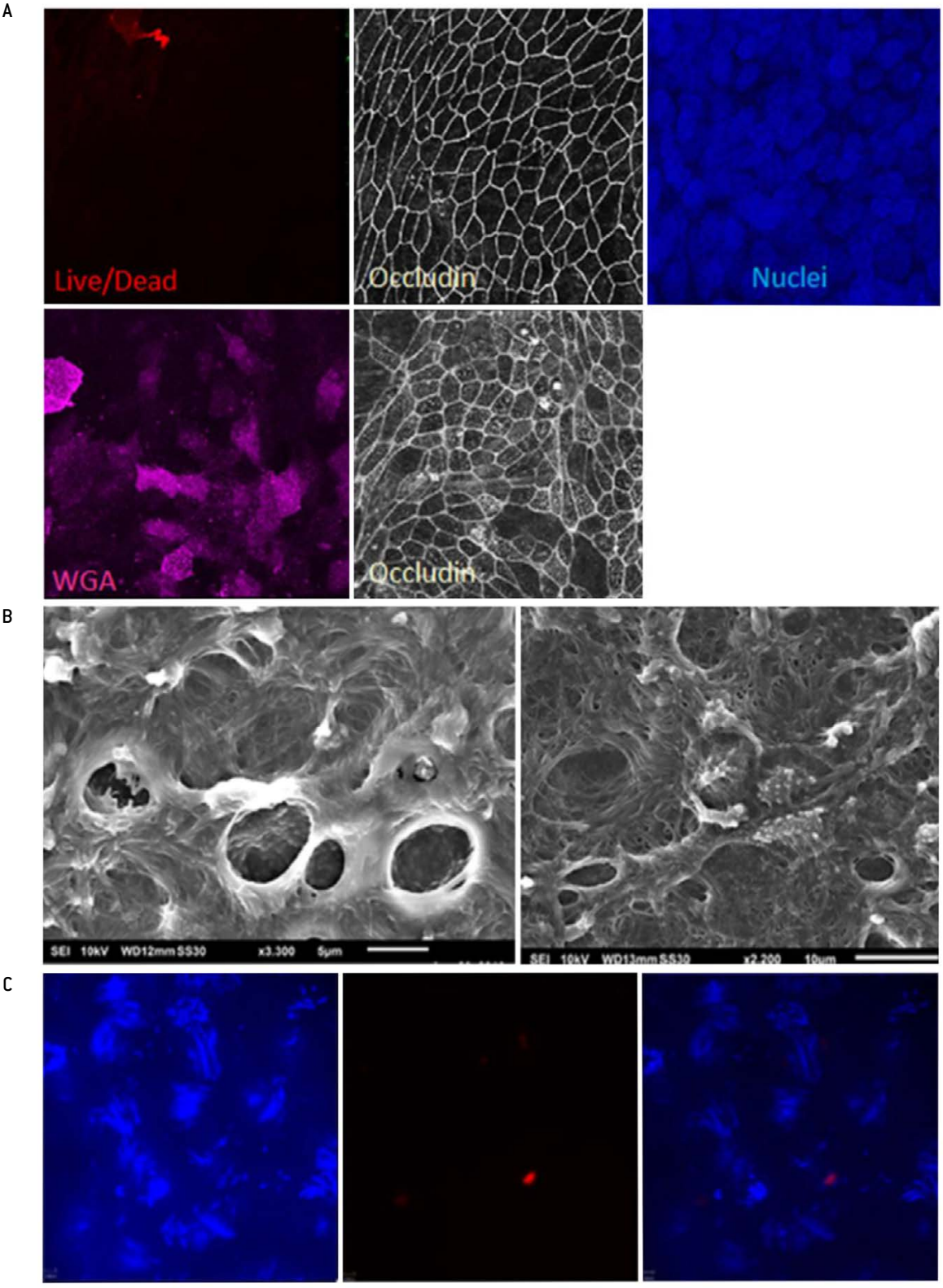
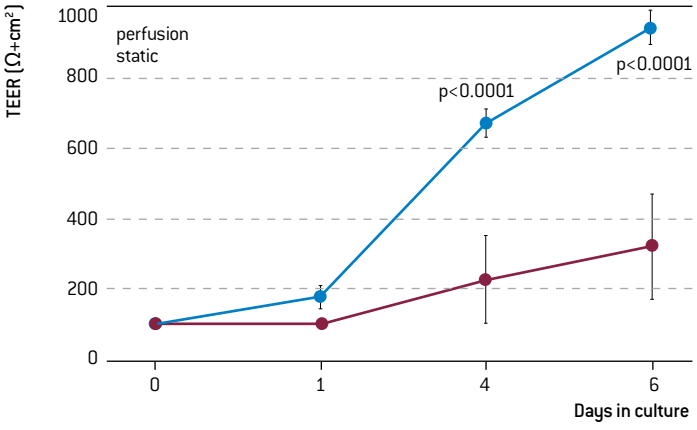


Figure 6

CLSM and SEM analyses of SAE cells grown under perfusion. (A) The upper panels depict CLSM analyses of SAE cells grown for 7 days under perfusion. Well-developed tight junctions (occludin, white) and cilia formation (WGA, lilac) are observable. High rates of live cells as illustrated by the limited number of dead cells that would appear red stained after the live/dead cell viability staining (Thermo-Fisher Scientific, red). Nuclei were stained 20 using Draq5 (blue). (B) The middle panel shows SEM of intact SAE epithelia grown for 7 days under perfusion. (C) Live cell imaging of SAE cilia movement upon stimulation with beads (snapshot). Cilia were stained using WGA (blue), latex beads are depicted in red.

increase in TEER was observed over time for the two conditions. Measurements on NHBE and SAE cells were taken on several days after ALI (Figure 7). TEER values obtained were corrected for the resistance and surface area of Transwell filters. TEER values of NHBE and SAE cells showed a constant increase over time in particular for cells grown under perfusion, and values of NHBE cells increased faster as compared to those for SAE cells (Figure 7). From day 21 onwards, TEER values in perfusion and static conditions were comparable and remained stable in long-term cultures (day 21: 1322.7 ± 89.5 Ω*cm² in perfusion, 1263.5 ± 76.7 Ω*cm² under static conditions). These experiments implied the integrity of the epithelial membrane during long-term culture under ALI and perfusion and illustrate the superiority of this system regarding accelerated epithelial cell development.

NHBE Cells



SAE Cells

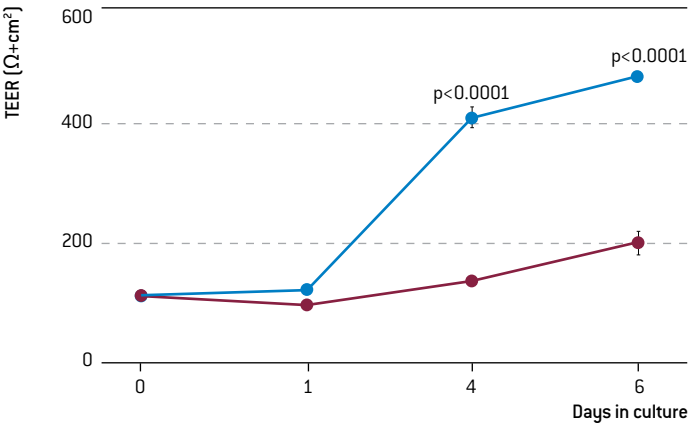


Figure 7
Comparison of TEER for NHBE and SAE cells cultured under static/perfused condition. Significantly higher TEER values were measured under perfusion in NHBE and SAE cells already after 4 and 6 days compared to static cultures. Each time point represents at least six values from three different Transwells and significant differences between the mean values of static vs. perfused conditions were analysed using Unpaired Student's T test.

Conclusion

In this Technical Note, we demonstrated that by using the Quasi Vivo® QV600 System the differentiation of both normal human bronchial epithelial and small airway epithelial cells *in vitro* can be greatly accelerated, thereby enabling fast-track addition of primary immune cells and significantly shortening of experimental procedures.

The growth of the epithelial cells in ALI and under perfusion demonstrated an improved and accelerated ciliogenesis and production of an intact muco-ciliary layer as well as improved barrier properties as compared to cells grown under static conditions. The fast-track development of highly differentiated lung epithelial cells due to applying perfusion significantly facilitates further applications such as studying airborne challenges, pathogen interactions[5], drug applications, or addition of novel compounds to monitor cytotoxicity or cell viability following exposure.

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