ADVANCED AIRWAY EPITHELIAL CELL CULTURE MODEL SYSYEMS USING ADHESIVES

DEVELOPING AND VALIDATING AN ADHESIVE-BASED CELL CULTURE TECHNIQUE FOR AIRWAY EPITHELIAL CELLS WITH APPLICATIONS IN CELL PATTERNING AND MICROFLUIDIC STUDIES

By

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Lay Abstract

This work describes a versatile and cost-effective cell culture method for micropatterning adherent cells on a porous membrane using pressure-sensitive double-sided adhesives. This technique allows growing cells using conventional methods, and it enables an easy transfer of the cultured cells on the membrane to a microfluidic chip. The adhesives can also be used to form different patterns of cultured cells, which can be used to create a separation between groups of cells to study cell migration. In this thesis, we evaluate the toxicity effect of four different adhesives on two airway epithelial cell lines. We show that the bonding between the wet adhesive and the microchannel device is strong enough to allow perfusion. The ease of use and cost-effective model has the potential to assist biomedical researchers with research questions that may be presently difficult to assess in standard cell culture systems.

Abstract

Airway epithelial cell culture is largely performed under static conditions in conventional cell culture dishes, plates or flasks. These models are easily accessible and user-friendly, widely being used in diverse discovery research and drug development applications. However, static culture models do not incorporate dynamic forces of airflow or interstitial fluid flow that are experienced *in vivo* by airway epithelial cells. Recent advances in airway epithelial cell culture are beginning to incorporate dynamic forces. However, these models are often proprietary, require access to specialized equipment, trained personnel and facilities, all factors that restrict such methods from being widely adapted by biomedical researchers. To address this application requirement and technology gap, we have developed and tested the use of adhesives in airway epithelial cell culture that enables us to create micropatterns, apply the technique to a cell migration study and develop an accessible microfluidic chip for perfusion that involves minimal equipment. Our results display data that supports using adhesives for advanced airway epithelial cell culture models that provide an easily accessible system for cell micropatterning and introduction of dynamic forces.

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List of Abbreviations and Symbols

AEC	airway epithelial cell
ALI	air-liquid interface
ASL	airway surface liquid
Calu-3	human epithelial lung adenocarcinoma cell line
CF	cystic fibrosis
COPD	chronic obstructive pulmonary disease
GFP	green fluorescent protein
DAPI	4’,6-diamidino-2-phenylindole
HAEC	human airway epithelial cell
HBEC	human bronchial epithelial cell
HBEC6-KT	human bronchial epithelial immortalized cell line
IL-1β	interleukin 1 beta
IL-6	interleukin 6
IL-8	interleukin 8
LDH	lactate dehydrogenase
OOAC	organ-on-a-chip
RCT	randomized control trial
SD	standard deviation
USA	United States of America

Declaration of Academic Achievement

I, Nicholas Tiessen, declare the work of this thesis to be of my own. I am the sole author of this document. I performed all of the research with the assistance of Mohammadhossein Dabaghi (Figure 12, 13), Yechan Kim, Cathy Cao and Abiram Chandiramohan (Figure 11) based out of McMaster University. The research was supported by federal and provincial funding held by my Principal Investigator, Dr. Jeremy A. Hirota.

Introduction

Human airway epithelial cells (HAECs) are largely cultured in static culture systems with flat surfaces. Although this method of cell culture is widely used, recent innovation in cell culture methods has exposed possible improvements to the conventional system that could be made to study a more physiologically accurate response to a tested stimulus. Perfusion and organ-on-a-chip (OOAC) models for airways or lung tissue have been able produce results that significantly differ from traditional static culture systems and that can replicate whole organ tissue responses[1–4]. Models such as these may provide outlets for unique drug testing conditions that are otherwise impossible in a traditional static culture plate, flask or dish. Further investigation and validation of these models may provide a platform to perform drug interventions that could bypass the need for animal studies and/or improve on the reproducibility of data when moving from models to clinical trials.

Advancements in HAEC culture have been made in recent decades that provide novel methods and conditions for studying these cells[3–8]. These models introduce forces and microenvironments, more closely representative of the native *in vivo* environment for HAECs to attempt to improve the translational application of *in vitro* cell studies to animal and human models. Yet, there are some features of using these models that have not facilitated their widespread uptake into biomedical research labs for preliminary testing. First, most models of HAEC perfusion culture or organ-on-a-chip models require pre-seeding the cells into a defined channel[4,7,9]. While this method is relatively simple, experimental possibilities are lacking in the system because of the pre-defined structure of the channels. The models also lack scalability and customization options that allow users

to easily modify variables to address specific aims of their experimental questions. In addition, after perfusion experiments, the cells are inaccessible which may be leaving behind key data such as protein expression level changes, cell morphology changes, or other effects from the introduced perfusion or stretch.

The following thesis outlines progress towards three objectives. It was of interest to determine the compatibility of using adhesives directly in cell culture, so cytotoxicity was of primary importance. Secondly, the adhesives were desired to be used in applications of cell patterning and assembly of a perfusion culture device. Our group has investigated the properties of four commercially available adhesives in airway epithelial cell culture model systems. We demonstrate that adhesives can be used as a cell culture method of micropatterning with applications in cell migration and modular assembly of organ-on-chip devices without prior cell seeding. The adhesive models are can be imaged during experimentation and deconstructed to allow access to cells post perfusion. In addition, the model may have future applications in primary airway epithelial studies as a model for personalized biological responses for improved therapeutic treatment options and exploration of use with additional adherent cell types such as endothelial cells.

Drug Development

Drug development process

Developing a drug is a complicated, slow and very strongly regulated process[10]. The usual process in Canada consists of five major stages (Figure 1)[11]. Stage 1 is drug discovery. In this stage, pharmaceutical companies and researchers begin initial testing

phases and perform screens that may screen anywhere from 5,000 compounds to several million compounds for several that achieve a desired biological effect. These compounds are deemed lead compounds, and the top lead compounds will move forward to Stage 2 of the process, preclinical studies[12–14]. The second stage requires thorough *in vitro* cell studies

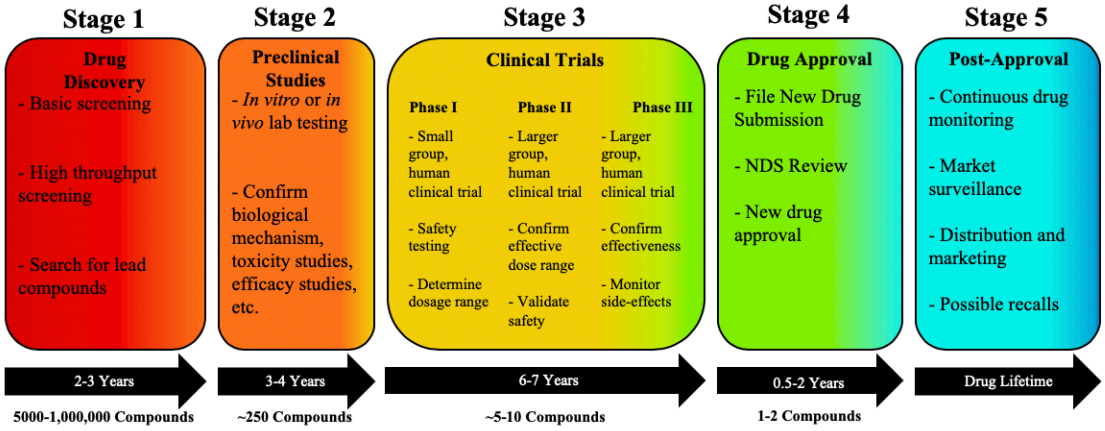


Figure 1: The drug development process stages.
An illustrative diagram of the five drug development pipeline stages. Each stage is displayed with the approximate number of compounds and years required to achieve one Health Canada approved drug.

and *in vivo* animal studies of the lead compounds for characteristics such as metabolism rate, excretion rate, specificity, safety, toxicity and adsorption[10,11,15]. The research in this stage is translational and the outcomes will inform the researchers which select compounds are worthy of the large financial investment that is required to begin a drug in clinical studies. Thus, this stage is crucial in the process because proper validation of a drug in stage 2 can prevent time and money being wasted from testing an ineffective drug in clinical studies of stage 3[16]. Furthermore, nearly 95% of drugs that enter Stage 3 human trials do not succeed, emphasizing the importance of the preliminary data collected in the preclinical experiments[16]. Stage 3 involves all aspects of clinical studies of the now handful of lead compounds. These experiments often incorporate the current benchmark method

for studying disease interventions, randomized control trials (RCTs)[17]. Phase I clinical trials involve small healthy volunteer populations (often <100) and will investigate the pharmacokinetics of the drugs. These initial tests also aim to find effective dose ranges and immediate serious side effects[11,17]. In Phase II, a larger group of diseased individuals are further tested to assess the potency of the drug. Further dose and safety tests would also be performed. Compounds with effective results and approved safety characteristics would then move to Phase III. Here, large studies with many individuals (often thousands) would be performed to compare to other treatment options and confirm effectiveness, dose ranges, safe usage and side effects[11,17,18]. Finally, Phase IV clinical trials (not shown in Figure 1) involve continuous monitoring of the drug after approval and market launch. These studies will help inform necessary recalls, adjustments to dosage range and long-term effects[11,18]. Out of the initial several thousand, or million compounds tested, often only one will make it through the process leading to a successful drug therapy. Stage 4 involves all of the drug file submission and approval processes. Then, in Stage 5, the drug is continuously monitored for long term effects, market surveillance and possible necessary recalls[11,17]. The entire process takes often greater than 10 years and was estimated to cost ~2.5 billion dollars in 2014[16,19].

Common pitfalls during drug development

Research that transitions from the *in vitro* models to *in vivo* animal or clinical studies is called translational research. Increasing efficiency in this translational period has been the target of significant research in the past years[14,16,20]. Hits that fail in proceeding from

this translational stage to successful clinical studies and eventual Health Canada or FDA approval can cost hundreds of millions of dollars. Therefore, developing translational methods to study drugs in that incorporate forces experienced in a physiological environment may have significant benefits for the drug development pipeline[16].

In addition, many common chronic diseases are highly variable and individuals often experience a broad range of effects from an approved drug[21]. For example, of the 10 best-selling therapeutic drugs in the USA, it was reported that 4 to 25 patients must be treated to yield one patient with a favourable response to the therapy[22]. In Seyhan's review of translational research (2019), they describe the full spectrum of basic research translating to medical practice and emphasize a range of this process deemed the *Valley of Death*. Within this region, data suggests that 80 to 90% of drug discovery projects fail before being tested in humans[16]. Furthermore, of those that proceed to clinical trials, approximately 50% of drugs fail approval in Phase III clinical trials. The exact cause of inefficiencies in this process varies with drug and disease pathway both as significant factors. However, advancements in *in vitro* studies and development of personalized medicine devices may provide significant improvements to the translational research involved in a getting a drug approved[14,16,20–22].

Advancement in cell culture techniques and limitations

One rapidly expanding area of research that is aiming to improve translational research is microfluidic technology used in cell culture methods[23]. OOAC models and microfluidic devices introduce forces that the cells tested would normally experience *in vivo*[3,4,7,8,24,25].

Furthermore, it is becoming increasingly common to see reports of new microfluidic devices being used for point-of-care diagnostics and medical research[26,27]. These models have been applied to function as several organ systems such as heart, nerve and lung on-a-chip models[4,7,28,29]. Lung-on-a-chip models and advanced HAEC culture models are an area of research appears promising as a method of streamlining the drug development process. It has the potential to lower money wasted on drugs that fail to translate to effective therapies in clinical trials. However, these models are not without their limitations. The models are limited in their flexibility of design[1,4,7]. They often require pre-seeding cells, restricting possible outcome measurements and they also limit access to the cells after perfusion. These are limitations that need to be addressed to increase the applicability and uptake of these modern and translational research techniques of cell culture.

Epithelial Cell Culture Techniques for Studying Lung Disease

Airway epithelial cell culture

The human airways are coated with epithelial cells. These cells provide a line of defense against inhaled pathogens and particulates[30]. HAECs are also involved in the phenotype of several airway diseases such as chronic obstructive pulmonary disease (COPD), asthma, idiopathic pulmonary fibrosis (IPF) and cystic fibrosis (CF)[31,32]. To improve on current treatment for patients affected with airway disease, drug studies are often performed on HAECs. The most widely adopted method of culturing HAECs is the submerged monolayer static culture system. Additional models and methods for cell culture exist and have shown data to support that growing in submerged monolayer conditions may restrict

HAECs from responding to stimuli as they would in their native environment *in vivo*[4,33]. Furthermore, the development of new engineered methods of adherent cell culture methods, such as micropatterning techniques, perfusion systems and organ-on-a-chip models provide unique approaches to HAEC studies that could are not possible in either submerged monolayer or traditional air-liquid interface culture methods[1,3,4,34].

Submerged monolayer cell culture

By in large, adherent cells including airway epithelial cells are grown on flat surfaces including well-plates, culture dishes, microscope slides, and culture flasks under submerged monolayer[33,35]. In these conditions, the cells grow adhered to the bottom surface of the culture surface and the feeding media is supplied in a thin layer on the surface of the cells. Although these platforms have been widely applied, they are limited to two dimensional (2D) rigid cell culture surfaces with low flexibility in design and architecture[35,36]. This technique in flasks or plates is useful for initial drug testing however, the experimental conditions are unable to integrate air exposure on the apical surface of the cells, dynamic forces associated with airflow or interstitial fluid flow – all conditions and forces that are observed *in vivo*. For instance, shear stress and mechanical strain have been shown to regulate biological pathways such as protein translocation and inflammatory response in HAECs[37,38]. Notably, in a lung-on-a-chip model, HAECs that were exposed to forces of basal fluid perfusion, apical air perfusion and stretch produced organ-level inflammatory response cascades when compared to whole, living lung tissue[4]. Such cascades are not producible in standard submerged static culture methods. Incorporating

dynamic conditions into airway epithelial cell culture to incorporate these physiological forces may be a crucial component for obtaining promising leads in the drug development pipeline.

Air-liquid interface culture

To address the lack of air exposure on the apical surface of HAECs during cell culture, devices such as Transwell® inserts are used as a standardized way to grow at ALI. This method utilizes a porous membrane that separates the cell monolayer in two compartments. The basal compartment feeds the cells through the membrane while the apical compartment is used to feed the cells in submerged monolayer until confluency is reached. Then, the apical media can be removed, and the cells continue to be fed from the basal compartment. Treating the cells this way has been shown to trigger cell differentiation which produces more physiologically relevant responses to stimuli[39–42]. This method has been recently adopted as a model for HAEC wound closure and migration and invasion assays[43,44]. Furthermore, this method introduces conditions that have been shown to induce a cell morphology more closely representative of an actual airway epithelium[45]. However, the transwell culture technique still fails to incorporate any perfusion of air or fluids on the cells.

Perfusion and organ-on-a-chip models

Even further advanced are perfusion and organ-on-a-chip models. These microfluidic culture systems often integrate forces of flow and mechanical strain on the cells that attempt to replicate conditions experienced *in vivo*[3,4,7,8]. In addition, biological responses of cells

grown in systems that incorporate such forces produce differing results when compared to cells grown in the conventional culture systems previously mentioned[3,4,7,8,46].

Perfusion models involve some method of flow with respect to HAEC culture. Several microfluidic devices for culturing HAECs have been developed that either incorporate perfusion of media, perfusion of air or both[1,2,47]. Blume *et al.* (2015) developed a model that to hold a commercially available transwell insert[1]. This model allows for cells to be grown conventionally in the transwell inserts, and once grown to confluency the transwell is moved to a customized perfusion device. The device perfuses media in the basal compartment to simulate interstitial fluid flow and air perfusion in the apical compartment to simulate breathing. The group tested their model against a static culture system using grass pollen, a known inflammatory stimulus. Interestingly, HAECs in the the stimulated perfusion condition produced a higher concentration of IL-8 than the stimulated static condition. Furthermore, the cytokine production of the perfusion control condition was greater than the stimulated static condition. Thus, development of advanced models that integrate forces more closely representative of a physiological environment processes may have significant impacts in our basic understanding of cell biology through to drug discovery.

OOAC models are even more advanced than perfusion models. One of the most influential progressions in this field was the development of a lung-on-a-chip[3,4]. The lung-on-a-chip incorporated epithelial cell growth on top of a porous membrane. Endothelial cells were adhered and culture on the bottom of the same membrane which introduced cell to cell interaction and signalling that would be experienced *in vivo.* On the sides of the

cellular growth chamber were vacuums that allow differential pressures to be introduced to the sides of the chip, thus stretching the growth membrane and introducing stretch that is mimetic of the human lung. The model was shown to cause increased uptake of nanoparticulates and subsequent transport into the basal endothelial cell component. Such results are also seen when testing whole mouse lung tissue which suggests that the microenvironment and forces applied to the cells in the lung-on-a-chip model may closely replicate organ-level function. Achieving responses in cell culture models that closer approach *in vivo* conditions could potentially offer an alternative platform to improve the results of animal or clinical trials of drugs in development.

However, most perfusion and OOAC models use a method of cell seeding into a channel with an incubation period that requires for adherence over time prior to experimentation[3,4,7,8]. This prevents the cells from being easily accessed in the device during or after perfusion. The cause of this characteristic is the pre-molded design of the culturing device. This also restricts the scalability and customization available to other researchers looking to implement similar devices into their experimental protocols. Therefore, despite the attempts to create advanced models of human airway epithelial cell culture, limitations currently still exist that need to be addressed to expand the applicability and uptake of these model systems.

Miniaturized cell culture and adherent cell patterning techniques

Miniaturized cell culture models provide suitable microenvironments to study live cells with a significant reduction in reagents and improved ability for high-throughput and

control of model design[35,48,49]. Advances in miniaturized cell culture fabrication techniques are now enabling investigation into cell-cell interactions and biological responses, while offering cell patterning capabilities by bypassing the constraints of conventional cell culture systems[34,35]. Many miniaturized cell patterning methods require costly and difficult manufacture techniques (*e.g.* stereolithography) that limit their translational application into the labs of biomedical researchers interested in studying the airway epithelium[50]. While this technique has high resolution (1-2μm), it demands access to a clean-room facility and specialized photolithography equipment[34,50,51]. A cost-effective, simple method of live cell micropatterning would allow for broad uptake of micropatterning methods to miniaturize current largely used static cell culture methods.

Many standardized cell assays have yet to be explored in the context of cell culture miniaturization. For example, a common assay used to study epithelial cell health and response is the wound-healing assay[52]. The method involves applying a physical scratch to the surface of a confluent culture of adherent cells growing at submerged monolayer and observing the migration of the cells as they *repair the wound*. Although the method has been well characterized, it lacks consistency due to individual differences in the application of the scratch. New automated wound application tools and culture insert products have assisted in making the wound-healing assay more reproducible[53–55]. Within the new wound application methods there are many that can be highly accurate including optical laser cell removal, electrical cell removal, physical barrier application and chemical cell removal. However, some of the features of using these techniques lend them unfavourable for broad uptake into biomedical research labs[44,53]. Physical disruption to the cells can cause

mechanical injury and release cytoplasmic contents into the surrounding media[56]. The extend of mechanical injury is difficult to control for these methods, so physical barrier approaches that do not cause injury to the cell can generate more reproducible data for cell migration assays, including wound-healing assays[53]. Within physical barrier products for cell migration studies, the options are fairly limited in their customization. A standardized method for accurate, reproducible cell migration studies with the ability to completely customize the experimental constraints could be beneficial for wound healing applications of epithelial cells in the future.

Overarching Hypothesis and Objectives

Overarching Hypothesis

Commercially available adhesives provide an accessible technology applicable to airway epithelial cell culture systems with applications in cell patterning and perfusion culture systems.

Objective 1: Characterize the impact of commercially available adhesives on airway epithelial cell viability in culture

In this thesis, I characterized the cellular viability of Calu-3 and HBEC6-KT cells grown in the presence of four commercially available adhesives both quantitatively and qualitatively.

Objective 2: Determine the feasibility of deploying commercially available adhesives for airway epithelial cell patterning and cell migration studies

In this thesis, I developed an accessible method for patterning airway epithelial cells using ARclean® 90716 adhesive and standard static cell culture conditions. Furthermore, the patterning technique was validated in a cell migration application.

Objective 3: Determine the feasibility of deploying commercially available adhesives for perfusion cell culture models of airway epithelial cells

In this thesis, I characterized physical characteristics of a novel adhesive assembled perfusion chip. The chip was validated to grow cells with perfusion for extended durations of time and produce measurable cytokine measurements to inflammatory stimuli.

Additional Note:

The following work does not conform to a traditional hypothesis testing-based thesis. Rather, this thesis is objective based, and the work was performed that was motivated by validation of the application of the tested adhesives. Thus, the data to follow follows the validation of these models and is not presented as hypothesis-driven as a traditional medical sciences thesis.

Materials and Methods

Reagents

Calu-3 human adenocarcinoma airway epithelial cells (HTB-55™, ATCC®, USA) were cultured in alpha minimum essential medium (αMEM, Corning®, USA) supplemented with fetal bovine serum (10%, FBS, WISENT Inc., Canada), and antibiotic-antimycotic (100U/mL penicillin, 100µg/mL streptomycin, 0.25µg/mL amphotericin B, Gibco®, USA) and HEPES buffer (10 µM, Gibco®, USA). HBEC6-KT cells were developed and provided by Dr. John D. Minna (UT Southwestern) and were cultured in Keratinocyte-SFM medium (KSFM, Gibco®, USA) supplemented with epidermal growth factor (EGF, 0.8ng/mL), bovine pituitary extract (BPE, 50µg/mL) and antibiotic-antimycotic (100U/mL penicillin, 100µg/mL streptomycin, VWR®, USA). Trypsin used (0.05%, 0.53mM EDTA in HBSS) was obtained from Corning®, USA. Recombinant Human IL-1β was obtained from Peprotech, USA (#200-01B). The IL-6 and IL-8 enzyme linked immunosorbent assay kits were obtained from R&D Systems, USA (#DY206 and #DY208, respectively). Trypan blue dye was obtained from ThermoFisher Scientific, USA. Lactate dehydrogenase (LDH) kit was obtained from ThermoFisher, USA (#C20301) The adhesives used were ARclad® 7535-12, ARclean® 90716, ARclear® 8932EE (Adhesives Research Inc., USA) and Scotch™ 7951 (3M®, USA). All adhesives were sterilized with ethanol (70%) and ultraviolet radiation for 20 minutes before use. Porous polyester membranes (0.4µm, transparent, #PET0413100) were obtained from Sterlitech, USA. Polydimethylsiloxane (PDMS) was obtained from Dow Chemical Company, USA. Fluorescent imaging was performed with Calcein AM viability dye (5µM, Life

Technologies®), Hoechst nuclear dye (20µg/mL, ThermoFisher Scientific, USA) and NucRed™ Live 647 ReadyProbes™ Reagent (2 drops/mL of PBS, Life Technologies®) diluted in phosphate buffered saline (PBS, Corning®, USA).

Epithelial Cell Culture

Calu-3 and HBEC6-KT cells were cultured in an incubator at 37°C with 5% carbon dioxide air on a two-day feeding cycle. For perfusion experiments, Calu-3 cells were perfused with media at a flow rate of 20µL/min for 24 hours. The entire microfluidic device was incubated at 37°C for the duration of perfusion.

Microscopy

All microscopic images were taken with the EVOS M7000 microscope using GFP, DAPI and CY5 light cubes (ThermoFisher, USA). GFP, DAPI and CY5 light cubes were used to visualize Calcein AM, Hoechst and NucRed™ Live 647 ReadyProbes™ Reagent dyes, respectively. Calcein AM is a colourless dye that is converted to a green-fluorescent Calcein molecule after live cells' cytosolic esterase enzymes cleave of the AM group. Therefore, it only stains live cells green-fluorescent. Hoechst and NucRed™ Live 647 ReadyProbes™ Reagent both stain the nuclei blue-fluorescent or red-fluorescent, respectively, of dead or alive cells.

Objective 1

Qualitative viability analysis

Viability qualitative analysis was performed on Calu-3 and HBEC6-KT cells using Calcein AM and Hoechst dyes.

Trypan blue viability analysis

After imaging the cells, 500µL of trypsin was placed on the cells and incubated for 10 minutes at 37°C. The resuspended cells were centrifuged at 1200 x g for 6 minutes and were resuspended with trypan blue for live/dead analysis in a Countess™ II Automated Cell Counter (ThermoFisher, USA). Data is expressed as:

$$\%\ Live\ Cells = \left(\frac{\#\ Live\ Cells}{Total\ Cells}\right)\ x\ 100$$

LDH analysis

The LDH assay was performed according to the manufacturer. Data is expressed as a percent of the high control LDH signal. In all cell types, LDH is a cytosolic protein that is found in nearly all living cell types[57,58]. When a cell dies and its cell membrane becomes permeable, LDH is released. Therefore, LDH can be quantified as a measure of cell death.

Objective 2

Cell patterning

Varying shapes of growth area were designed in Silhouette Studio V4 software and cut out of ARclean® 90716 with a Silhouette CAMEO® 3. A polyester membrane was adhered between two adhesives and attached to a culture plate. The top adhesive included the cut-out pattern, and the top adhesive seal remained on. HBEC6-KT cells were grown until confluency was achieved, then the top adhesive seal was removed. The chips were incubated with Calcein AM at 37°C for 20 minutes and microscopy was performed.

Cell migration application

Cell migration was chosen as an application for cell patterning by creating slits that had a width of 600µm and were cut into ARclean® 90716 with a Silhouette CAMEO® 3 with gaps of 600µm between each other. The adhesives were adhered to a cell culture plate with the top adhesive seal remaining on. HBEC6-KT cells were grown to confluency on the patterned slits, and the top adhesive seal was removed. Additional conditions were introduced of 15% FBS and 20% FBS following adhesive seal removal. Every 24 hours, microscopy of the cells was performed with after incubating the cells with Calcein AM for 20 minutes at 37°C.

Objective 3

Perfusion model fabrication

The microfluidic model consists of two layers of adhesive, a cell growth membrane and a molded lid made of PDMS adhered together in a sandwich-like manner to a glass slide that provides optical clarity for microscopy, and structural support of the chip as shown in Figure 2A. The mold for the PDMS lid was fabricated by conventional photolithography using a cleanroom facility. PDMS was prepared and cured on the top of the mold according to the manufacturer's directions. Before pouring PDMS to the mold, two pieces of silicone tubing were placed on the designated located as each chip inlet and outlet. This technique was applied for all functional experiments using adhesive assembled chips.

For all of the functional experiments, a 5 mm diameter circular growth area was cut out of the top adhesive. The workflow for adhesive chip assembly involves several steps, as presented in Figure 2B. The process begins with cutting ARclean® 90716 on a Silhouette CAMEO® 3 (Figure 2B1). Both seals are removed from the bottom adhesive, and only the bottom seal is removed from the top adhesive. A porous polyester membrane is adhered between the two layers of adhesive and compressed with a hand roller (Figure 2B2). Following cell growth to confluency (Figure 2B3), the top seal of the top adhesive is removed, and the molded PDMS lid is adhered to the chip (Figure 2B4). Then, the assembled chip with cultured cells was ready to be perfused (Figure 2B5).

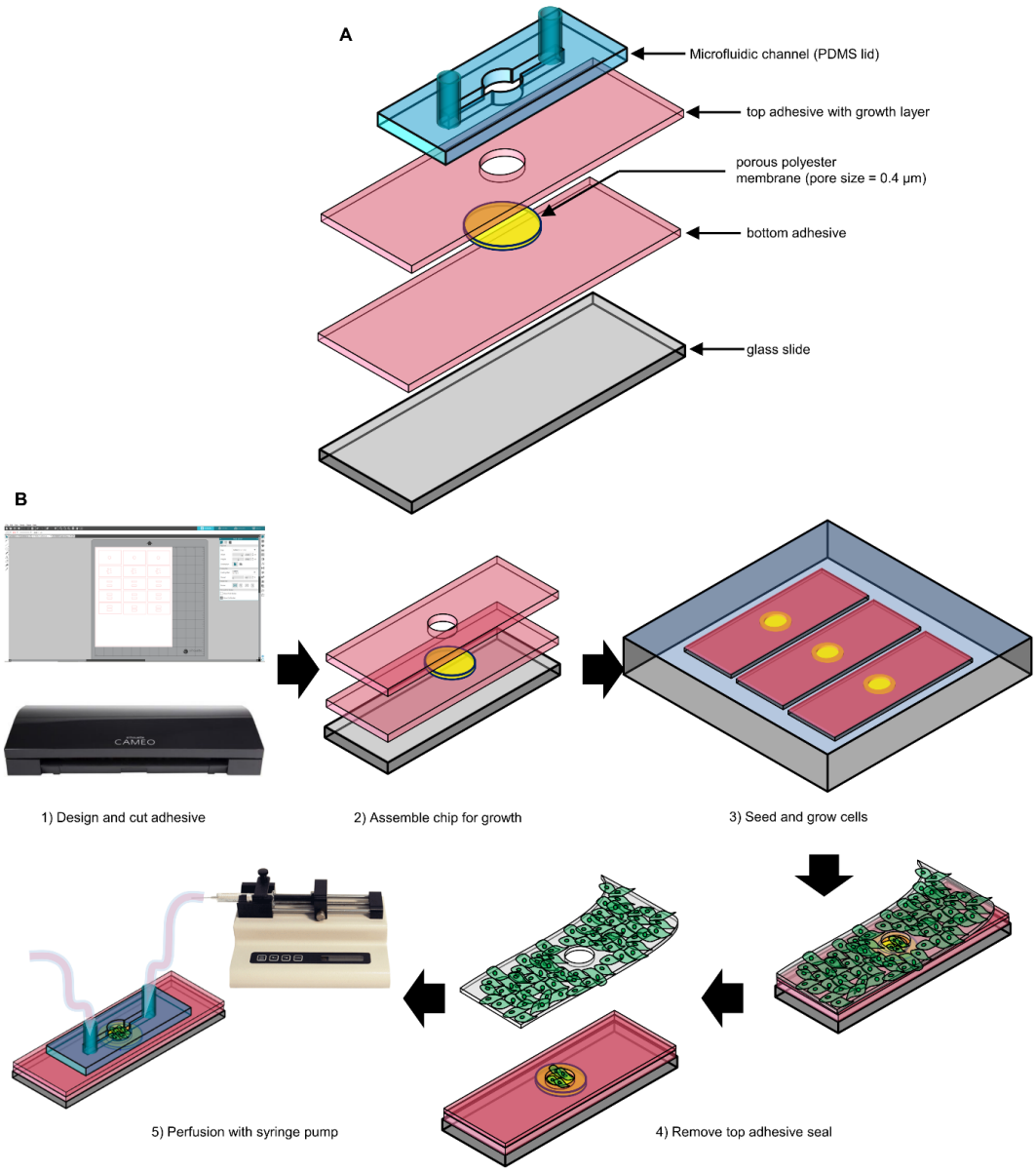


Figure 2: Adhesive based epithelial cell perfusion chip manufacture process and workflow.
(A) Explosion diagram of the stacked adhesive chip, including all layers. (B) Basic design methods and workflow for assembling and growing epithelial cells in the adhesive based chip.

Flow rate validation

Chips were submerged in αMEM at 37°C for 1 week. Following submersion, chips were removed from media the top adhesive seal was removed, and the PDMS lid was adhered. Chips were connected to a syringe infusion pump (Harvard Apparatus, USA), with water run through, effluent collected, and mass measured. Chips were subjected to rates of 10, 50, and 250 μL/min for 30 minutes at each rate. Effluent mass was recorded at 5-minute intervals for a measure of volume.

Burst pressure measurement

Chips were submerged in αMEM 37°C for 1, 3, or 7 days. Following submersion, the top adhesive seal was removed, and the PDMS lid was adhered. The chips were connected to a syringe pump with a pressure transducer included to monitor system pressure over time (USB Output Pressure Transducer, Cat# PXM409-USBH, OMEGA Engineering Inc., USA). A block was inserted into the output of the chip, and the syringe pump was set to 500μL/min and run in the chip until burst pressure was achieved. The final burst pressure was recorded.

Targeted cellular growth

Calu-3 cells were grown on adhesive masks with 5 mm diameter circles. Images of the cells were taken with Calcein AM when the cells grew to confluence. The top adhesive lining layer was removed, and images of the cells were taken again with Calcein AM.

Perfusion viability

Calu-3 cells were grown on adhesive assembled chips as shown in the workflow of Figure 2B. The cells were stained with Calcein AM and NucRed™ Live 647 ReadyProbes™ reagent and imaged. The top adhesive lining layer was removed and the PDMS lid was adhered to the chip. The chip was connected to a syringe pump and perfused 20µL/min of media over the cells for 24 hours. The PDMS lid was removed and the cells were imaged again as described previously. Calu-3 cells were chosen over HBEC6-KT cells for all perfusion experiments because of their ability to maintain tight junctions for several days after reaching confluency[59,60]. In addition, they provide robust cytokine release profiles that are more favourable to measure at more dilute concentrations that will be obtained from collecting large volumes of effluent media in the perfusion chip[60,61].

IL-1β stimulation

Calu-3 cells in chips were either perfused with standard αMEM supplemented as previously described, or αMEM containing IL-1β (5ng/mL). The chips were perfused at a flow rate of 20µL/min for 24 hours.

ELISA analysis

Calu-3 cells grown in a static 96-well culture plate were grown with either control or media containing IL-1β (5ng/mL) for 24 hours. Calu-3 cells grown in the adhesive assembled perfusion chip were perfused control media or media containing IL-1β (5ng/mL) for 24 hours at a flow rate of 20µL/min. Due to the large difference in total media

collected, the IL-6 or IL-8 release that was represented as a concentration (pg/mL) was converted to total IL-6 or IL-8 (pg). For static culture conditions, total collected volume was 200µL. For perfusion culture conditions, total collected volume was 28.8mL (20µL/min*60min*24hr). The total cytokine released was calculated by applying the following formula:

$$Total\ Cytokine\ (pg) = [ELISA\ Raw\ Value\ (pg/mL)][Collection\ Volume\ (mL)]$$

Simulated velocity profile and shear stress

Velocity and shear stress profiles of an ARclean® 90716 adhesives were generated with COSMOL Multiphysics software following the reported method from Dabaghi *et al.* 2020[62].

Results

Objective 1

Adhesive viability analysis

First, the effect of commercially available adhesives on cell viability was assessed. This is an essential step to determining a suitable adhesive for the future experiments because the adhesives would be submerged in culture media for cell culture. Qualitative visual analysis of Calu-3 cell and HBEC6-KT viability with four commercially available adhesives did not display any significant changes in viability, as shown with Calcein AM viability dye, or cell morphology (Figure 3A). There was a significant decrease in the percentage of viable HBEC6-KT cells grown with ARclear® 8932EE (52.3% ± 25.3) when compared to a control (91.5% ± 2.9)($p=0.037$). For the remaining conditions, qualitative analysis of total viable cells grown with the four adhesives did not yield any significant difference between the adhesive-grown conditions in comparison to a control (Figure 3B and C). In addition, LDH assay results confirmed that adhesives did not compromise the cell viability (Figure 3D and E).

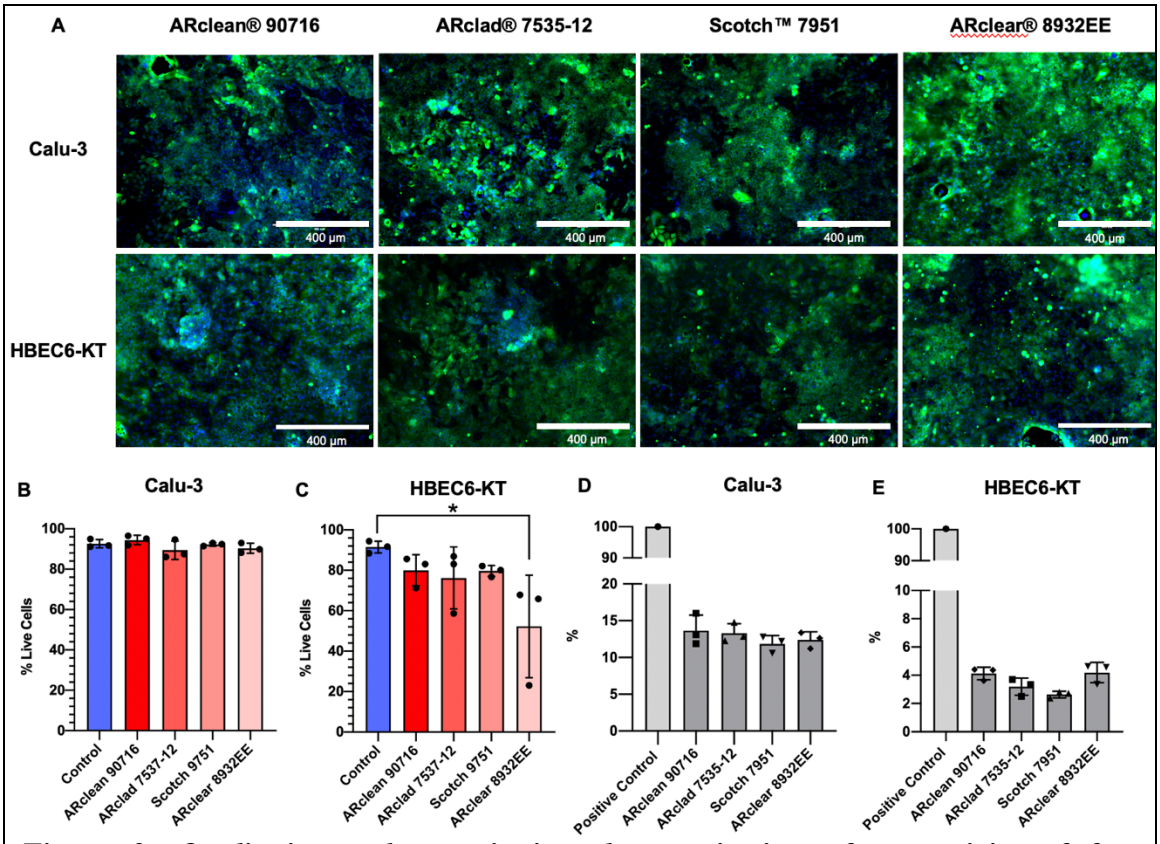


Figure 3: Qualitative and quantitative characterization of cytotoxicity of four adhesives using both Calu-3 and HBEC6-KT cells.

(A) Calu-3 and HBEC6-KT cells grown on top of ARclad® 7535-12, ARclean® 90716, Scotch™ 7951, or ARclear® 8932EE adhesives, respectively. The following growth to confluency, the cells were incubated in PBS with Calcein AM viability dye (green) and Hoechst nuclear dye (blue) at 37°C for 20 minutes. Images were taken at 100X magnification, and scale bars in the bottom right corner represent 400 µm. (B and C) Percent of viable cells following adhesive exposure, as determined by trypan blue live/dead stain. (D and E) LDH assay after growth for 7 days adhesives expressed as a percentage of a lysed positive control. Graphs are expressed as mean ± SD. One-way ANOVAs were performed with a Tukey's post hoc test for multiple comparisons ($P<0.05$*). n=3

Objective 2

Cell patterning

We have tested HBEC6-KT cells seeded on ARclean® 90716 adhesive to better understand the resolution of applying the adhesives to cell patterning. HBEC6-KT cells successfully remained adhered to the culture plate after removing the top adhesive seal and maintained the selected shapes (Figure 4). The cells were shown to maintain a defined structure to a resolution of 1mm.

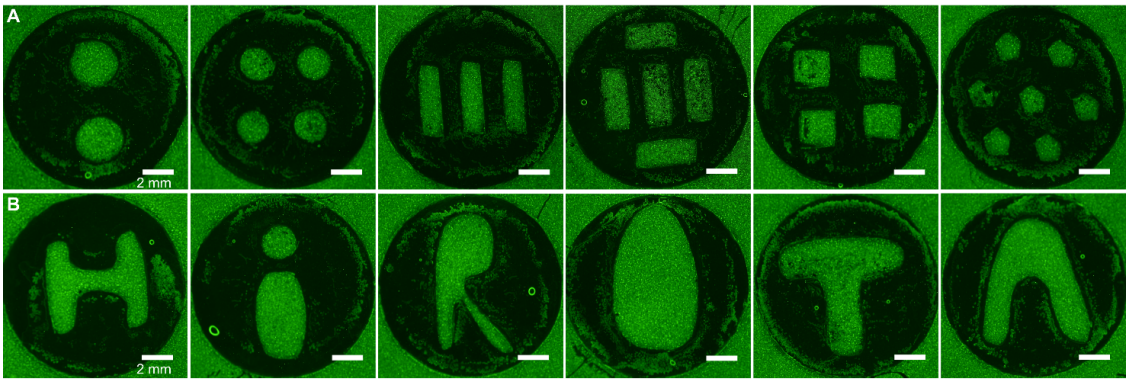


Figure 4: Xurography cell patterning of HBEC6-KT cells using ARclean® 90716 adhesive.
HBEC6-KT cells were grown on patterned ARclean® 90716 adhesive chips and grown to confluency. The adhesive seal was removed, and the cells were stained with Calcein AM viability dye (green) in PBS. (A) Various geometric shapes and (B) HIROTA cut out of ARclean® 90716. Scale bars represent 2mm.

Cell migration application

HBEC6-KT cells were seeded on the patterned adhesives that were adhered to the bottom of a 12-well plate and were grown to their confluency to test them in a cell migration application. Then, the top lining layers with grown cells were peeled off, and a gap without cells (Figure 5A) was created, resulting in the formation of two slits of cultured cells. The patterned cells were maintained for several days until the cells patched the artificially

created gaps, as seen in Figure 5B. Figure 5C shows a close-up view of HBEC6-KT cells that fully covered the gap.

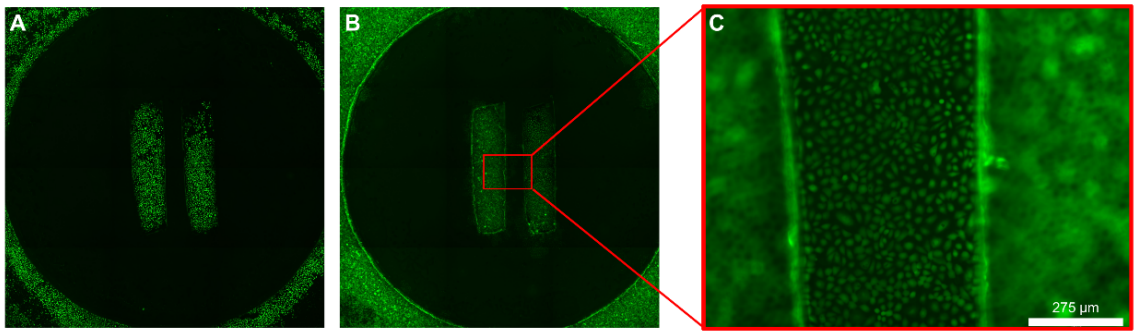


Figure 5: Cell migration application of adhesives using HBEC6-KT cells and ARclean® 90716 adhesive.
(A) HBEC6-KT cells were grown to confluency on ARclean® 90716 adhesives with two 600µm slits cut in it. The adhesive seal was removed, and the cells were stained with Calcein AM at Day 0. (B) HBEC6-KT following 7 days of cell culture. (C) A magnified image of the HBEC6-KT cells growing in the central region between the two 600µm growth slits.

Objective 3

Perfusion model physical characteristics

Since the patterned adhesive with growing cells was kept under a wet condition (submerged in a media to maintain the cells) and they were still wet (even after peeling off the liners) upon bonding to the PDMS lid, it was essential to evaluate the quality of this "wet" bonding between the wet adhesive and the PDMS lid. First, we conducted a flow experiment to ensure that there was no flow leakage.

The proposed adhesive assembled microfluidic model remained structurally viable after 30 minutes of several flow rates (10, 50, and 250µL/min) after being submerged for 1 week, simulating cell culture conditions (Figure 6A). Calculated flow rates from the device were

9.76 ± 0.043, 48.19 ± 0.41 and 251.30 ± 0.66 µL/min for the 10, 50 and 250 µL/min set flow rates, respectively (± SD, n=3).

Next, the burst pressure of the assembled chips that were fabricated using a similar method was measured. The model withstood burst pressures of 328.33 ± 38.55, 244.67 ± 93.93 and 254.33 ± 137.04 mmHg for 1, 3, and 7 days of submersion, respectively (± SD, n=3, Figure 6B).

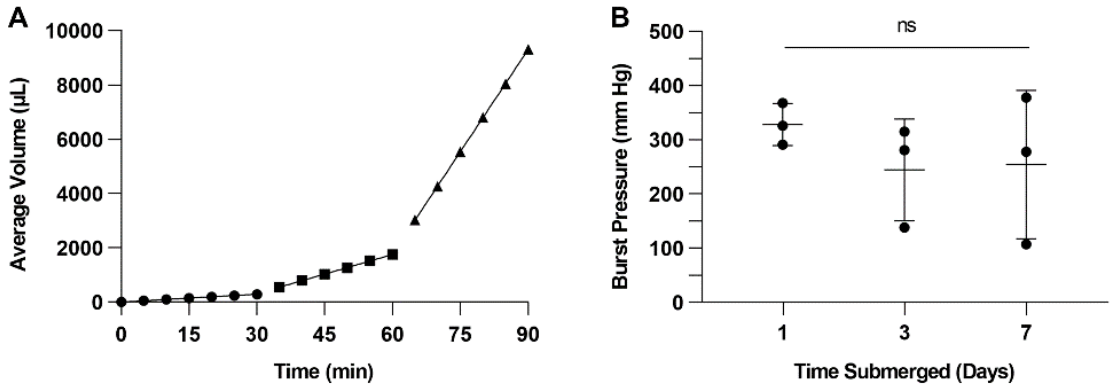


Figure 6: Volumetric flow validation and burst pressure analysis of ARclean® 90716 assembled perfusion chip.

(A) Collected volume of water flowed through adhesive assembled chips submerged in αMEM for 1 week at 37°C. Water was flowed through the chip with a syringe pump for 30 minutes at 10 (circles), 50 (squares) and 250 (triangles) µL/min. Lines represent segmented linear regression of each flow rate. Bars for SD were plotted but were too small to be shown (n=3). (B) Burst pressure of adhesive assembled chips following submersion in growth media for 1-7 days. Burst pressure is shown for all replicates of the three conditions, with bars showing SD and mean of the groups (n=3).

Table 1 – Quantitative measures to complement Figure 6 (±SD).

Volumetric Flow Validation				
Set Flow Rate (µL/min)	*Time (min)*	*Average Cumulative Weight (g)*	*Average Converted Volume (µL)*	*Calculated Flow Rate (µL/min, ±SD)*
10 µL/min	5	0.048	47.8	*9.76 ± 0.043*
	10	0.097	96.9	
	15	0.146	145.9	
	20	0.195	194.6	
	25	0.242	241.6	
	30	0.294	293.9	
50 µL/min	35	0.548	548.4	48.19 ± 0.41
	40	0.805	804.6	
	45	1.027	1026.8	
	50	1.266	1265.8	
	55	1.520	1519.7	
	60	1.758	1758.2	
250 µL/min	65	3.033	3033.4	251.3 ± 0.66
	70	4.270	4269.7	
	75	5.527	5526.6	
	80	6.811	6811.0	
	85	8.039	8039.1	
	90	9.311	9311.0	

Burst Pressure Analysis		
Days Submerged	*Burst Pressure (mmHg)*	*Average Burst Pressure (mmHg, ±SD)*
1	368	328.3
	291	
	326	
3	138	244.7
	281	
	315	
7	278	254.3
	278	
	107	

Perfusion model cell culture

To utilize the perfusion model for cell culture purposes, the maintenance of cells in a targeted growth area was desired to prevent cells from decreasing adherence of the PDMS lid to the top adhesive layer. Our proposed method of restricting Calu-3 cells in a targeted growth area (5mm diameter circle) was reproducibly successful in maintaining a defined interface of cell presence within the target area after adhesive seal removal (Figure 7).

In addition, it was essential that the cells could remain alive for several hours in the perfusion device under the force of flow, as these would be the conditions for an experiment in the device. Calu-3 cells were quantitatively demonstrated to remain viable, as shown with Calcein AM viability dye, in the adhesive assembled device after perfusion of media for 24 hours (Figure 8B) when compared to the same imaging protocol before perfusion (Figure 8A). NucRed™ Live 647 ReadyProbes™ Reagent was used to stain total nuclei as a visual reference to compare dead cells (red nucleus) to live cells (red nucleus and green cytoplasm).

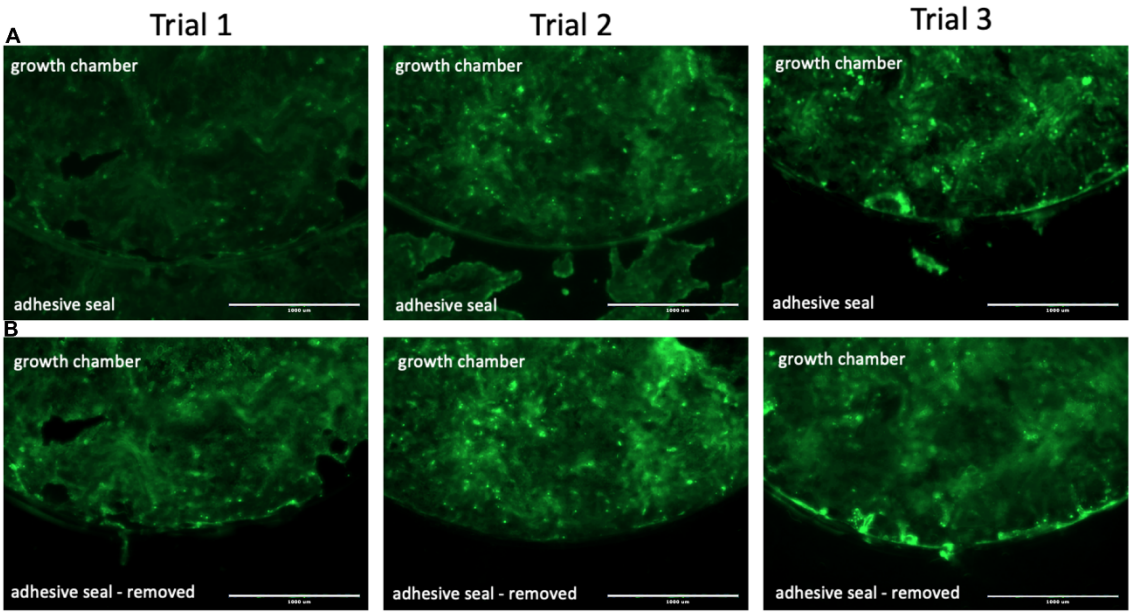


Figure 7: Maintenance of Calu-3 cellular growth in the targeted growth chamber of an adhesive assembled perfusion chip following adhesive seal removal.

Adhesive assembled chips were made with ARclean® 90716 with a 0.5cm diameter growth chamber. Calu-3 cells were seeded into the culture dish and grown to confluency. The cells were incubated with Calcein AM in PBS. The chips were imaged with a GFP light cube prior to removing the adhesive seal and after the adhesive seal was removed. Cells remained in the target growth chamber after seal removal. Images were taken at 40X magnification, and scale bars represent 1000 µm.

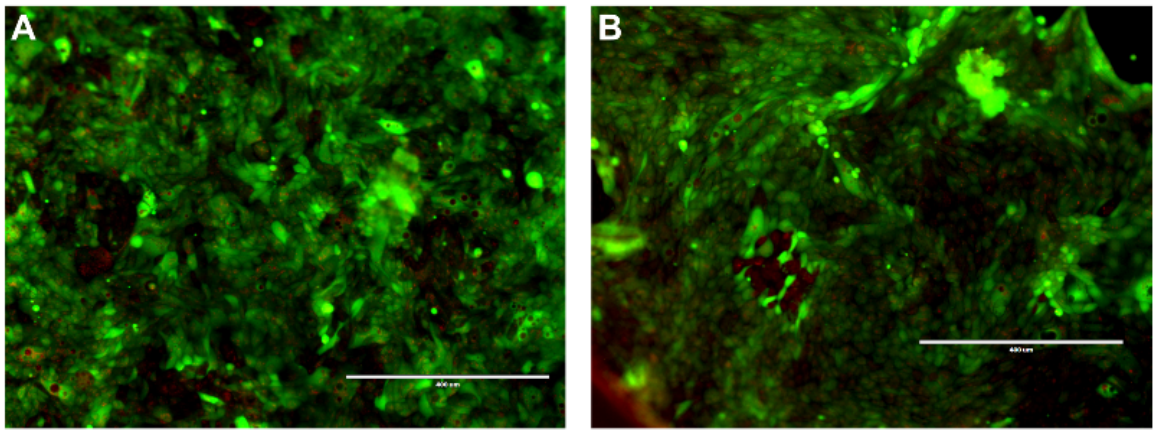


Figure 8 - Qualitative Calu-3 cell viability following growth with media perfusion in an ARclean® 90716 adhesive assembled chip.
(A) Cells were grown to confluency prior to perfusion of media and stained with Calcein AM viability dye (green) and NucRed™ Live 647 ReadyProbes™ Reagent (red) in PBS. (B) Cells following 24 hours of media perfusion at a flow rate of 20µL/min, stained as previously described. Images were taken at 100X magnification, and scale bars represent 400 µm.

Functional application of perfusion model

Calu-3 cells were used to determine if a known inflammatory cytokine release after stimulation with IL-1β (5ng/mL) would elicit different responses in static or perfusion culture systems, when compared to controls. The experimental outcome was assessed using the total IL-8 or IL-6 release (pg) to account for different culture volumes used between the static condition and perfusion condition. The release of IL-8 increased 12-fold when comparing the IL-1β stimulation conditions of static and perfusion cell culture ($p<0.01$)(Figure 9B). In the perfusion culture system, IL-1β stimulation produced a 9-fold increase of IL-8 compared to control perfusion ($p<0.01$). In both the static and perfusion IL-1β stimulation conditions, IL-6 release increased significantly compared to their

respective controls ($p<0.0001$)(Figure 9D). The perfusion model did not elicit an increased IL-6 release in the IL-1β condition when compared to the static IL-1β condition.

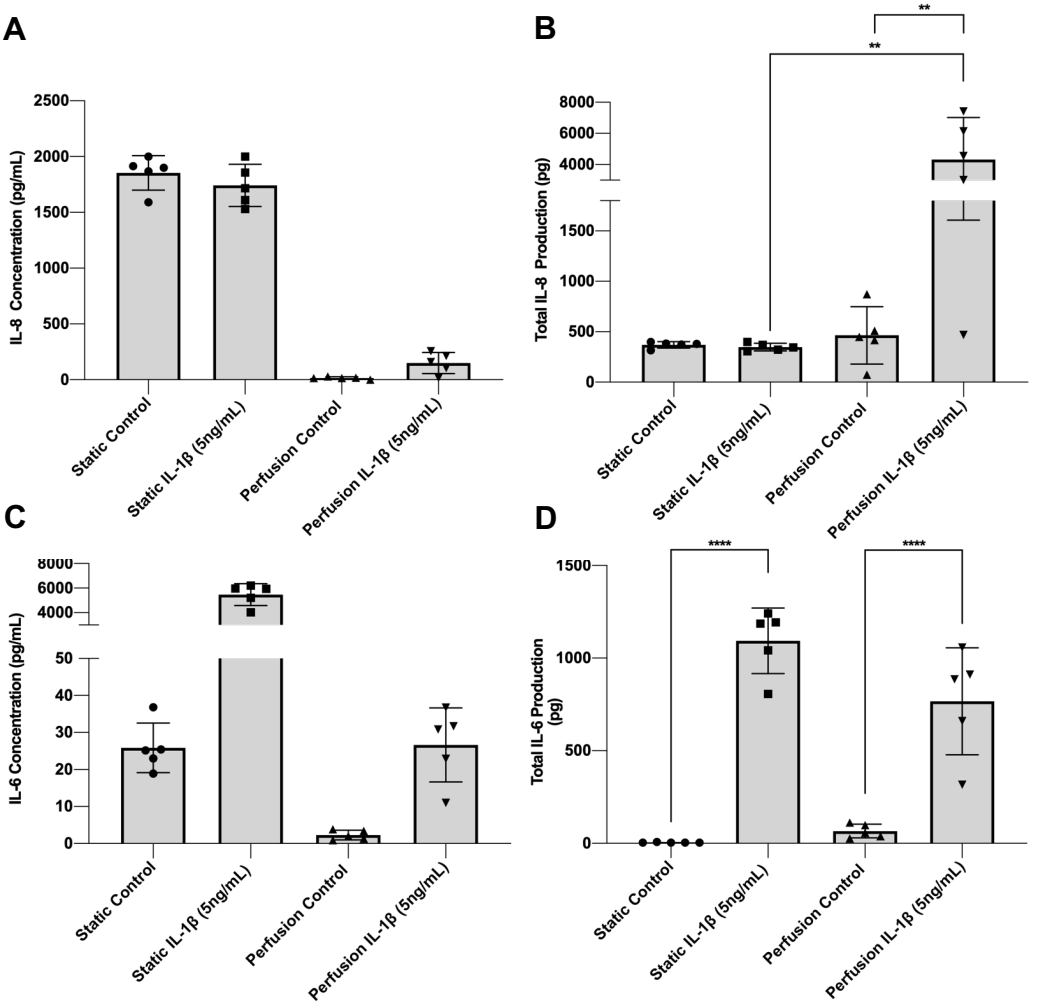


Figure 9 - Comparison of Calu-3 IL-6 and IL-8 release after stimulation with IL-1β in a static or adhesive assembled perfusion cell culture system.
(A) IL-8 concentration (pg/ml) after exposure of Calu-3 cells to control media, or IL-1β (5ng/mL) conditioned media for 24 hours in a static or dynamic cell culture system. (B) IL-8 release expressed as total IL-8 (pg) calculated by adjusting for total exposure media volume. (C,D) IL-6 concentration and total IL-6 production, respectively, as described above. Results are displayed as mean ± SD. One-way ANOVAs were performed with a Tukey's post hoc test for multiple comparisons. ($P<0.01$**, $P<0.0001$****). n=5

Table 2 – Quantitative measures to complement Figure 9 (±SD).

IL-8 Experiment			
Conditions	*Average IL-8 Concentration (pg/mL, ±SD)*	*Collection Volume (mL)*	*Average Total IL-8 (pg)*
Static Control	1854.3 ± 155.7	0.2	370.9 ± 31.1
Static IL-1β (5ng/mL)	1743.6 ± 188.7	0.2	348.7 ± 37.7
Perfusion Control	16.1 ± 9.9	28.8	464.6 ± 284.2
Perfusion IL-1β (5ng/mL)	150.0 ± 94.2	28.8	4319.1 ± 2711.7
IL-6 Experiment			
Conditions	*Average IL-6 Concentration (pg/mL, ±SD)*	*Collection Volume (mL)*	*Average Total IL-6 (pg, ±SD)*
Static Control	25.9 ± 6.7	0.2	5.1 ± 1.3
Static IL-1β (5ng/mL)	5470.4 ± 886.7	0.2	1094.1 ± 177.3
Perfusion Control	2.3 ± 1.3	28.8	66.6 ± 37.4
Perfusion IL-1β (5ng/mL)	26.6 ± 10.0	28.8	767.0 ± 288.5

Discussion

The drug development process requires several thousand or even millions of compounds to go through rigorous testing to produce an approved drug[11,12]. One of the major bottlenecking areas where compounds fail to move forward as an eventual therapy is the translation of data seen in *in vitro* studies to safe results in a human clinical trial[16,17]. It has been estimated that nearly 95% of compounds that are tested in human clinical trials fail to move forward to a manufactured drug[16]. Improving translational science techniques in lab research would benefit this process and save costs that are associated with failed lead compounds[14,16,21]. It has been shown that integrating forces in cell culture that are similar to forces HAECs experience *in vivo* produces differing responses to tested stimuli when compared to conventional methods[39–42]. Thus, widespread uptake of new cell culture models may have significant implications improving the drug development pipeline efficiency[16].

Current research in the field of airway epithelial cell biology has seen recent advances with cell culture miniaturization to incorporate cell patterning techniques that improve on throughput, cut costs of reagent use and provide researchers with more customization options[34,35]. In addition, significant progress has been made on microfluidic organ-on-a-chip devices that integrate forces into cell culture, which are ignored when studying cells in conventional static culture systems[3,4,7,8]. While these methods provide alternative options for researchers to study airway epithelial cells, they have limitations. Many miniaturizations and cell patterning methods require technically difficult procedures and access to equipment and training that is not readily accessible to biomedical researchers.

Furthermore, currently studied organ-on-a-chip models often require pre-seeding cells into a pre-defined structure cast out of a mold[1,4,7]. These models offer little flexibility in design and do not allow researchers to access the cells after perfusion culture to examine the effects of the desired stimuli. Our data suggested that using commercially available adhesives has several applications to address the aforementioned limitations. We showed that adhesives could pattern cells to a reasonable resolution (1mm, Figure 4) and may have applications in studying cell migration (Figure 5). In addition, we demonstrated a method of developing a microfluidic culture device using the adhesives that may be grown initially in a submerged monolayer culture before applying perfusion (Figure 2). The model was shown to have robust characteristics (Figure 6), maintain cell viability with perfusion for 24 hours (Figure 8), and demonstrate a promising application for studying dynamic airway epithelial inflammatory response (Figure 9).

Objective 1

Adhesive selection

Several commercially available adhesives were tested for applicability in cell culture and other applications. Although no significant cytotoxic effects were found between the four different tested adhesives (Figure 3), some limitations were discovered that led to the pursuit of using the acrylic-based ARclean® 90716 adhesive for cell patterning and perfusion model development. The following information represents qualitative findings that were made when doing preliminary testing with the adhesives. In testing, acrylic-based Scotch™ 7951 lost tactile properties following prolonged submersion in fluid, rendering it

unusable for cellular growth in submerged media. Polyester-based ARclad® 7535-12 was functionally suitable for experimental use but displayed autofluorescence when performing some fluorescent microscopy imaging, specifically in the DAPI light cube wavelength range. Silicone-based ARseal® 8932EE has very elastic properties, causing it to poorly retain the desired shape when it was cut and applied to surfaces. It also did not remain functionally tactile following prolonged submersion in media. Finally, ARseal® 8932EE was the only adhesive that showed a significant decrease in cell viability compared to a control, so it was not selected for further experimentation (Figure 3C). ARclean® 90716 fulfilled the desired properties for applications in microfluidic device manufacturing. After prolonged submersion in media, the adhesive remained tacky and it is made a robust acrylic plastic film coated in adhesive, so it was a strong candidate for being cut by a craft cutter. For these reasons and because there were no significant changes in cell viability for ARclean® 90716 (Figure 3), it was selected as the adhesive for all experiments.

Cell line selection

Both the Calu-3 cell line and HBEC6-KT cell line were already well-defined *in vitro* models in the Hirota lab. Although this influenced the initial testing of each cell line for translational function in the adhesive applications, there are properties of each cell line that make them quite suitable for use in the described conditions. HBEC6-KT cells were developed by a group Dr. John Minna's group and are a minimally immortalized HBEC line[63]. They provide robust immune responses to known airway stimuli and do not form extremely tight junctions[63,64]. The moderate strength junctions lend this cell line well to

generating defined cell patterns with the adhesive cut-outs (Figure 4,5). In addition, HBEC6-KT cells have been used for cell migration studies previously that lend them well to applying to a migration study with adhesives[65]. Calu-3 cells are a human lung adenocarcinoma cell line. Specifically, the cells are derived from the bronchial submucosal glands which are a plentiful source of secretory compounds in the lung. Thus, Calu-3 cells represent a strong model for performing experiments that may require any collection of mucins, airway surface liquid (ASL) or immune compounds such as cytokines[66–70]. These characteristics make Calu-3 cells suitable for verifying a functional application of a device, as was performed in Figure 9. In addition, Calu-3 cells grow well differentiated cultures at ALI which may benefit the applications of the adhesive assembled chip in the future [45,60].

Adhesive viability analysis

Until recently, adhesives had been used for manufacturing cell culture devices, but they had not been required to come in direct with the cells[71]. Our perfusion model required the adhesives to be submerged in culture media during the growth phase. Therefore, it was essential to verify that the adhesives were not toxic to either the HBEC6-KT or Calu-3 cell line. Both Calu-3 and HBEC6-KT cells did not show any qualitative changes in cell morphology or viability (Figure 3A). Although the HBEC6-KT cells appear to more uniformly cover the surface in the images, it must be noted that Calu-3 cells are known to grow and form 3-dimensional structure as they reach confluence. Regions that appear dark on images of Calu-3 cells (Figure 3A) in fact have cells emitting fluorescence in the dark regions. However, to expose the cells in these regions, the highest-grown cells would be

overexposed and saturate the image. It would be recommended for further applications like this, to use confocal fluorescent microscopy to improve the resolution at all planes of the image. In this experiment, Hoechst nuclear dye was used to stain all nuclei so that any cells that fluoresced blue, but not green would represent dead cells and would qualitatively suggest some cell death as a result of the adhesive in the culture plate. There were no visible instances of this circumstance.

To further validate the viability of cells grown with adhesives, two quantitative assays were performed. First, a trypan blue stain was performed on trypsinized Calu-3 cells that had been cultured on the tested adhesives, or just the plate alone. Although the trypsinized cell suspension was neutralized to prevent the trypsin from permeabilizing the membrane, a control condition with cells grown on the plate alone was included to obtain a baseline cell viability measurement. Trypan blue dye is able to enter the cell when the cell membrane is permeable, a characteristic of a dead cell[72]. In testing, all conditions yielded similar results, where no significant change in viability was found with the four adhesives. However, HBEC6-KT cells grown with ARclear® 8932EE showed a significant decrease in cell viability in the trypan blue assay when compared to control cells (Figure 3C). Although the data points for the ARclear® 8932EE condition are much more variable than the other conditions, the adhesive was not used for future experiments for this reason. The exact chemical composition of the ARclear® 8932EE adhesive is unknown, however it consists of a polyester film that supports the silicone adhesive on either side. Silicone itself has been shown to be relatively inert and not toxic in many cases[73,74]. Yet, in some instances, variants of silicones (or siloxanes) have been shown to cause negative effects[75].

In one low molecular weight form, siloxanes have been shown to change the structure of lipid bilayers in human skin[76]. Although this has not been replicated in HAEC cells and the exact composition of the silicone adhesive in ARclear® 8932EE is not known, it is possible that the adhesive elicits a change in the permeability of lipid membranes of HBEC6-KT cells. Alternatively, it is possible that the HBEC6-KT ARclear® 8932EE condition was exposed to trypsin for too long causing the membrane to permeabilize.

An LDH assay was also performed to determine if changes in viability could be measured from an alternative biological outcome. LDH is a cytoplasmic enzyme produced by all cells in the human body[57]. It is released into the supernatant of cell culture when cell membranes become permeable which makes it a useful marker for cell viability. There was no significant difference in the LDH release for either HBEC6-KT or Calu-3 cells (Figure 3D,E). The difference seen in the trypan blue assay was not reproduced in the LDH assay with samples from the same cell culture. In normal circumstances, the described experiments would be repeated to elucidate the effect of ARclear® 8932EE on HBEC6-KT cells. However, other adhesives being tested had more favourable physical characteristics and did not express any toxic effects on either HBEC6-KT or Calu-3 cells. As previously mentioned, ARclean 90716 was a favourable due to physical characteristics that made it more applicable for future applications of the adhesive. The viability data presented in Figure 3 provided strong evidence that using the adhesives in cell culture would not impact cell viability.

Objective 2

Cell patterning

To determine if it was possible to create small patterned cut-outs in the adhesive using a low-cost craft cutter, several shapes were cut out of ARclean® 90716 and used in HBEC6-KT cell culture (Figure 4). The smallest effective resolution of shapes that can be cut using this method is 1mm (Figure 4). However, cells may be grown in cut out areas and smaller measured distances can be created between the populations of cells. Using this method, the smallest effective resolution of distance between cut-outs is 600µm (Figure 5). It should be noted that distances of 500µm have been achieved but are inconsistent and led to more failed cuts (data not shown). Using a similar patterning technique, but a more precise and technically difficult method of creating the patterning mask, resolutions of 20µm have been achieved for live cell patterning of human B lymphocytes[51]. This resolution is significantly smaller than what our proposed method is able to achieve. However, the process of patterning with adhesives is simple and immediately accessible to biomedical researchers. In contrast, the method described in Wu *et al.* (2018) requires access to a precise photolithography device to generate the patterns. This acts as a barrier to biomedical researchers from testing hypotheses that may require cell micropatterning[35]. In our method, cells could be accurately grown to spots of 1mm. This could be suitable for applications in live cell microarray generation. Our method's low barrier to access and reasonable resolution may provide an easy-to-use method of cell micropatterning that could be applied readily to groups without access to equipment for such experiments.

Cell migration application

To confirm the application of the adhesive based cell patterning technique in an experimental application, cells were grown in two regions separated by a 600µm gap to mimic a cell migration study (Figure 5). In our testing, Calu-3 cells required approximately a week to cover the gap between the cells. This data was not reproduced or quantified to provide reproducibility. However, the time to cover the gap was consistent in three separate replicates, with only one representative replicate being shown in Figure 5. In future experiments, it would be of interest to attempt to quantify the time to grow across the introduced gap over several replications to validate the consistency of the system.

One of the most frequently tested applications of cell migration is a wound-healing assay[52]. The wound-healing assay would be a logical extension of the cell migration application that was validated with the adhesive mask technique in this work. In these assays, time to close the introduced gap, or "wound", is compared between experimental conditions and controls[52]. The original wound-heling assays were performed by scratching the cell surface with a pipette tip[77]. This method is extremely easy to adopt into a lab, but is understandably inconsistent, causing differing data between labs[52,77]. New devices and inserts have been invented to help standardize this protocol. However, the solutions to the pipette scratch variability do not offer the user any customization. Currently available methods for variable wound application are still limited in their scalability and some methods such as thermal wound application and laser wound application may introduce biological variability to experiments, as the amount of damaged cells that release factors to

surrounding cells is variable[54,78]. Thus, the adhesive migration application has potential to be used as a cost-effective wound healing tool that does not require causing serious mechanical damage to the cells.

Objective 3

Perfusion model physical characteristics

Our proposed microfluidic model involves a straightforward manufacturing process and design that was user friendly to assemble (Figure 2). The chip is made of materials that are applicable to microscopy and other imaging techniques because of its optically clear structural components and transparent membrane. Based on the application, the design and size of the chip can be easily modified without impacting the fabrication process. In other reported perfusion systems, this level of customization is not available without significant model design updates including developing new molds[1,4,7]. The functional components of the model include adhesives, a transparent polyester membrane, and a PDMS lid that are all optically practical with little interference in the wavelengths necessary for basic cellular imaging, with the exception of some adhesives. The model is also adhered to a glass microscopy slide, which, while being optically clear, also offers structural support to improve handling the chip and makes the chip easily insertable into the majority of microscope imaging platforms. Thus, the model has potential applications in studies monitoring real-time changes in cell morphology, migration, and gene/protein expression if used in a temperature and gas-controlled microscope.

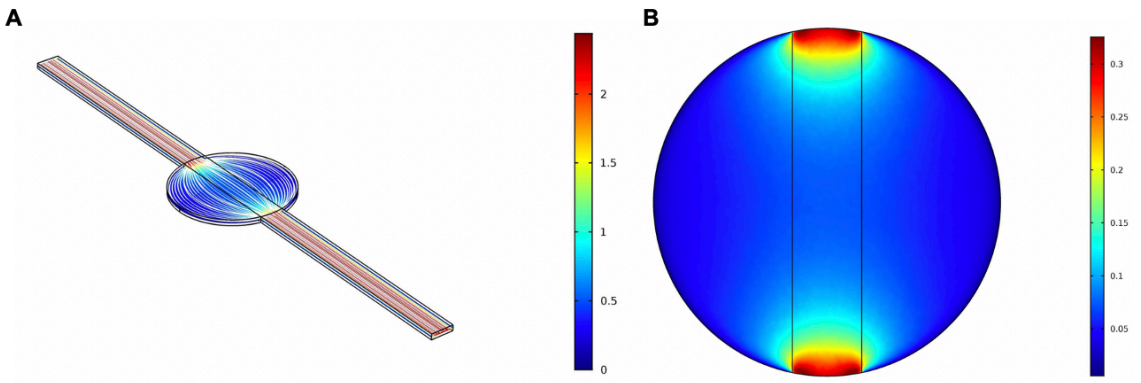
ARclean® 90716 was able to maintain a fluid-tight seal following submersion in media for one week at flow rates from 10-250 µL/min (Figure 6A), which exceed the necessary flow rate for experimental purposes. Therefore, the model is capable of undergoing increased fluid flow rates to test extreme shear stress responses on cells. The model was shown to be capable of withstanding burst pressures of 328.33 ± 31.48, 244.67 ± 76.69, and 254.33 ± 111.89 mmHg for 1, 3 and 7 days of submersion, respectively (Figure 6B). For applications of studying interstitial fluid flow, this well exceeds the typical human lung interstitial fluid pressure of 8 mmHg[79]. It was noted that the variation of burst pressures did vary as time submerged increased. Therefore, for using the perfusion chip, it is recommended to culture the cells at a relatively high seeding density to shorten the time required to grow the cells to confluency.

Cell culture and functional application of perfusion model

A beneficial feature of the adhesive based chip is its ability to avoid seeding cells into a channel prior to perfusion, as required by many reported epithelial chip-based culture models in the current literature[4,7]. Our proposed model allows cells to be cultured in submerged monolayer prior to being assembled with the PDMS lid for perfusion. The top seal of the top adhesive was left adhered to the chip during submerged culture. After growth to confluency, the chip was removed, and the top seal of the top adhesive was removed. We were able to contain cellular adherence to the 0.5cm targeted growth chamber reproducibly by using this technique (Figure 7).

Calu-3 cells were shown to remain viable after growing in the assembled chip for 24 hours of perfusion at a flow rate of 20 µL/min (Figure 8). Although the model remains tightly adhered during perfusion applications, the PDMS lid was easily removed after perfusion, allowing the cells to be stained again for imaging. This feature of the chip increases possible applications of study after exposing cells to dynamic forces. Based on previous reports of the effect of shear stress on the airway epithelium at near physiological shear stress levels, a flow rate was set to fall within the range of known shear stress levels that induce biological responses[2,80]. Flow of 20µL/min was simulated to cause a range of 0.05-0.3 dyn/cm^2 of shear stress in the chip, with the majority of cells growing experiencing <0.1 dyn/cm^2 (Figure 10B). IL-1β is known to induce expression of IL-6 and IL-8 cytokines with some testing being performed on Calu-3 cells[81–84]. Our model replicated these findings (Figure 10). Previously it has been shown that perfusion cell culture can elicit an increased cytokine released in response to an inflammatory stimulus when compared to a static system[1]. This finding was replicated with IL-1β induced IL-8 release (Figure 9A,B). More specifically, the perfusion IL-1β condition averaged more than a 10-fold increase in total IL-8 production (Figure 9B, Table 2). Therefore, it is possible that the characteristic response to the cells is dramatically different when the cells are experiencing perfusion culture. The exact pathway for this observation is not known. One suggested possible cause is a negative feedback loop.

Figure 10 – Simulated flow pattern and shear stress of the adhesive assembled perfusion chip at a flow rate of 20µL/min.
(A) Simulated fluid velocity perfused through the channel of an ARclean® 90716 assembled chip. The colour gradient represents the relative flow rate of fluid perfused through the chip (mm/s) at a set rate of 20µL/min. (B) Simulated shear stress of the centre of the channel of an adhesive assembled chip. The colour gradient represents the relative shear stress (dyn/cm^2) at a set flow rate of 20µL/min.



When cells are cultured in static submerged monolayer conditions, the molecules they produce collect in the supernatant media until the next feeding cycle. When the Calu-3 cells were grown in the adhesive perfusion chip, the media was constantly being replaced with fresh media, and removing the supernatant media. Therefore, the IL-8 that was being released may have been removed from the perfusion, allowing the cells to produce more. The same phenomena was not seen for IL-6 release of the same samples (Figure 9C,D). Yet, in both the static and perfusion culture conditions, IL-1β stimulated an increased release of IL-6. Further replication of this result should be tested in the future.

Conclusively, the model is suitable for studying inflammatory stimulus within the scope of our analysis. The increased IL-8 when comparing the IL-1β stimulated static and

perfusion conditions may suggest that biological responses with perfusion may differ significantly from cells grown in submerged monolayer. Perhaps the perfusion condition's biological responses more closely mimic human *in vivo* responses and could have translational applications in streamlining the drug development pathway.

Limitations and Future Directions

Limitations

The adhesive-based cell culture techniques described in this work are user-friendly and simple methods to use but they do require some special considerations when in use. Firstly, when cutting the adhesives with the craft cutter, the sharpness of the blade is essential. When blades began to slightly dull it causes the protective seal layers to lift and renders the cut unusable. For purposes of cell patterning, Calu-3 cells were problematic due to their tendency to form tight barriers[39,60]. The tight barriers provide a robust model for testing cells in a dynamic system however, this must be considered when using Calu-3 cells in small growth areas and greater success was seen when using the cells below 100% confluency to prevent allowing the cells to begin differentiation and significant airway surface lining fluid production.

Initial designs of the perfusion chip included only one adhesive that held the porous membrane to the glass slide. When testing this model, fluids that were perfused over the membrane would loosen the adhesive seal to the glass slide and cause a leak. The design was modified to sandwich the porous membrane between two adhesives, which provides more a much tighter seal and provides more structural support. Finally, when applying the

PDMS lid to the perfusion chip, it was noticed that cells can be physically stripped off of the porous membrane if too much force is applied. The integrated fluidic channel moulded into the PDMS lid is very shallow and excess force can cause the channel to collapse and lift the cells.

To use the adhesive assembled perfusion chip, minimal equipment is required. The Silhouette CAMEO® cutter is inexpensive and requires minimal training. In addition, using a syringe pump to perfuse is relatively accurate and easy to use as well. However, many incubators do not support power supply inside, so perfusion experiments were performed on the benchtop and temperature was controlled with a water bath. This feature of the method makes performing the experiment very simple but may have impacts on sterility so caution should be taken when dealing with the cells outside of an incubator. For this reason, the cells were cultured with a combination of antibiotic and antimycotic.

Future Directions

Future directions of the patterning adhesive culture technique include developing a method to quantify cell migration in the adhesive migration application experiments. It is of interest to be able to use the adhesives and perform cell migration assays. We are also interested in applying the cell patterning technique towards developing a live cell microarray platform that would utilize the same adhesive and principles described in this work. This could provide an accessible method that is similar to currently reported options for testing numerous replicates of independent live cell culture samples all in one well of a culture plate[85,86].

In regard to the perfusion chip, there are several avenues of future exploration that could advance the chip's applications. First, it is of interest to incorporate primary HAEC samples within the different culture techniques. These preliminary experiments were planned for this spring but were postponed due to the pandemic shutdown. Expanding the current finding into primary HAEC culture would only further increase the possible implications of using this model for translation science research and future application in personalized medicine. We are also interested in introducing a complexity to the chip design in which a perfusion channel could be introduced on the bottom to facilitate ALI perfusion culture. A rendered example of this advancement can be seen in Figure 11. Not only could this model

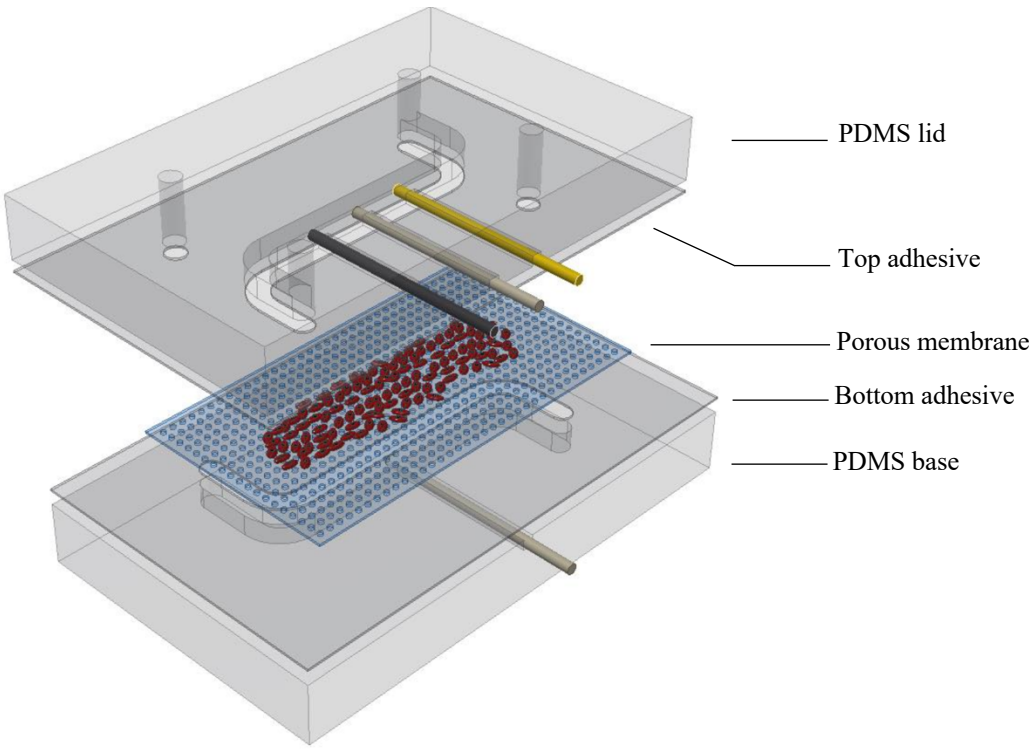


Figure 11 – Schematic explosion diagram of a rendered adhesive based ALI perfusion chip.
The model involves the same core structure as the adhesive perfusion chip. A PDMS top lid has an integrated channel for airflow on the cells, while the bottom PDMS base has

a channel for media perfusion. The model also has rendered channels for microwires (black, grey and yellow rods).

facilitate perfusion culture at ALI, we could introduce endothelial cells to the bottom of the porous membrane and the perfusion chip would have characteristics of an organ-on-a-chip model for airway immune response.

It is also of interest make further advancements in the customization of the perfusion chip. New designs will be tested to determine new applications that could be studied with perfusion forces. For example, a cell migration assay could be cut into the centre growth chamber in the perfusion chip, and cell migration time could be assessed under flow. Furthermore, scaling the chips to be able to include several perfusion channels that requires only one inlet would allow several more chips to be run simultaneously. Finally, the Hirota group has done some recent validation of microwires that can sense ions such as chloride and bicarbonate. We will integrate wires in the perfusion chip so real-time measurements of ion release could be monitored with perfusion. This would allow for precise data of drug responses to be measured. An example of applying this would be to culture airway epithelial cells of a patient with CF and use the perfusion chip to determine which drug treatment options provide the greatest modulation in chloride.

Conclusion

The drug development pipeline involves considerable troubleshooting and hits often fail in transitioning from lab-based experimental models to *in vivo* models. Several attempts have been made to introduce forces that more closely replicate physiological conditions to improve cell culture research at the *in vitro* level. However, these models often have barriers in their use that prevent their uptake into a broad range of biomedical research labs. In this thesis, we have developed and tested an adhesive-based cell culture system that enabled us to pattern cells on a membrane using a patterned-cut adhesive as a mask. First, we showed that these adhesives were not toxic and did not compromise cell morphology. We created various patterns on adhesives and could grow cells onto them. The cultured cells on these patterned adhesives were imaged after removing their top lining layers and transferring the desired patterns to the cells. This patterning method was utilized to form an array of slits for a cell migration application. Moreover, this method was used to grow cells in a targeted area and was attached to a microfluidic chip for perfusion. The chips were perfused with an inflammatory stimulus and an increase in cytokine was seen. The results suggest that this method can be used to grow cells outside of a microfluidic chip, which can be transferred later to a microfluidic channel for further analysis.

References

1. Blume, C. *et al.* Temporal monitoring of differentiated human airway epithelial cells using microfluidics. *PLoS One* (2015). doi:10.1371/journal.pone.0139872
2. Zhu, Y. *et al.* Baseline goblet cell mucin secretion in the airways exceeds stimulated secretion over extended time periods, and is sensitive to shear stress and intracellular mucin stores. *PLoS One* (2015). doi:10.1371/journal.pone.0127267
3. Huh, D. A human breathing lung-on-a-chip. in *Annals of the American Thoracic Society* (2015). doi:10.1513/AnnalsATS.201410-442MG
4. Huh, D. *et al.* Reconstituting organ-level lung functions on a chip. *Science (80-.).* (2010). doi:10.1126/science.1188302
5. Huh, D., Hamilton, G. A. & Ingber, D. E. From 3D cell culture to organs-on-chips. *Trends in Cell Biology* (2011). doi:10.1016/j.tcb.2011.09.005
6. Xu, Z. *et al.* Application of a microfluidic chip-based 3D co-culture to test drug sensitivity for individualized treatment of lung cancer. *Biomaterials* (2013). doi:10.1016/j.biomaterials.2013.02.045
7. Benam, K. H. *et al.* Small airway-on-a-chip enables analysis of human lung inflammation and drug responses in vitro. *Nat. Methods* (2016). doi:10.1038/nmeth.3697
8. Niemeyer, B. F., Zhao, P., Tuder, R. M. & Benam, K. H. Advanced Microengineered Lung Models for Translational Drug Discovery. *SLAS Discovery* (2018). doi:10.1177/2472555218760217
9. Konar, D., Devarasetty, M., Yildiz, D. V., Atala, A. & Murphy, S. V. Lung-On-A-

Chip Technologies for Disease Modeling and Drug Development. *Biomed. Eng. Comput. Biol.* (2016). doi:10.4137/becb.s34252

10. Robuck, P. R., Wurzelmann, J. I. & Merrick, M. Understanding the drug development process. *Inflamm. Bowel Dis.* (2005). doi:10.1097/01.MIB.0000184851.46440.a3
11. SPharm. The drug review and approval process in Canada. (2020).
12. Tonkens, R. An overview of the drug development process. *Physician Exec.* (2005).
13. Hughes, J. P., Rees, S. S., Kalindjian, S. B. & Philpott, K. L. Principles of early drug discovery. *British Journal of Pharmacology* (2011). doi:10.1111/j.1476-5381.2010.01127.x
14. Mohs, R. C. & Greig, N. H. Drug discovery and development: Role of basic biological research. *Alzheimer's and Dementia: Translational Research and Clinical Interventions* (2017). doi:10.1016/j.trci.2017.10.005
15. McNamee, L. M., Walsh, M. J. & Ledley, F. D. Timelines of translational science: From technology initiation to FDA approval. *PLoS One* (2017). doi:10.1371/journal.pone.0177371
16. Seyhan, A. A. Lost in translation: the valley of death across preclinical and clinical divide – identification of problems and overcoming obstacles. *Transl. Med. Commun.* (2019). doi:10.1186/s41231-019-0050-7
17. Umscheid, C. A., Margolis, D. J. & Grossman, C. E. Key concepts of clinical trials: A narrative review. *Postgraduate Medicine* (2011).

doi:10.3810/pgm.2011.09.2475

18. Aronson, J. K. What is a clinical trial? *British Journal of Clinical Pharmacology* (2004). doi:10.1111/j.1365-2125.2004.02184.x
19. Mullin, R. Cost to Develop New Pharmaceutical Drug Now Exceeds $2.5B. *Scientific American* (2014).
20. Goldblatt, E. M. & Lee, W. H. From bench to bedside: The growing use of translational research in cancer medicine. *American Journal of Translational Research* (2010).
21. Zeggini, E., Gloyn, A. L., Barton, A. C. & Wain, L. V. Translational genomics and precision medicine: Moving from the lab to the clinic. *Science (80-.).* (2019). doi:10.1126/science.aax4588
22. Schork, N. J. Personalized medicine: Time for one-person trials. *Nature* (2015). doi:10.1038/520609a
23. Song, P., Hu, R., Tng, D. J. H. & Yong, K. T. Moving towards individualized medicine with microfluidics technology. *RSC Adv.* (2014). doi:10.1039/c3ra45629c
24. Yager, P. *et al.* Microfluidic diagnostic technologies for global public health. *Nature* (2006). doi:10.1038/nature05064
25. Jodat, Y. A. *et al.* Human-Derived Organ-on-a-Chip for Personalized Drug Development. *Curr. Pharm. Des.* (2019). doi:10.2174/1381612825666190308150055
26. Pandey, C. M. *et al.* Microfluidics Based Point-of-Care Diagnostics.

Biotechnology Journal (2018). doi:10.1002/biot.201700047

27. Natarajan, S., Su, F., Jayaraj, J., Shah, M. I. I. & Huang, Y. A paper microfluidics-based fluorescent lateral flow immunoassay for point-of-care diagnostics of non-communicable diseases. *Analyst* (2019). doi:10.1039/c9an01382b
28. Ribas, J. *et al.* Cardiovascular Organ-on-a-Chip Platforms for Drug Discovery and Development. *Appl. Vitr. Toxicol.* (2016). doi:10.1089/aivt.2016.0002
29. Huval, R. M. *et al.* Microengineered peripheral nerve-on-a-chip for preclinical physiological testing. *Lab Chip* (2015). doi:10.1039/c4lc01513d
30. Lambrecht, B. N. & Hammad, H. Allergens and the airway epithelium response: Gateway to allergic sensitization. *Journal of Allergy and Clinical Immunology* (2014). doi:10.1016/j.jaci.2014.06.036
31. Crystal, R. G., Randell, S. H., Engelhardt, J. F., Voynow, J. & Sunday, M. E. Airway epithelial cells: Current concepts and challenges. in *Proceedings of the American Thoracic Society* (2008). doi:10.1513/pats.200805-041HR
32. Barratt, S., Creamer, A., Hayton, C. & Chaudhuri, N. Idiopathic Pulmonary Fibrosis (IPF): An Overview. *J. Clin. Med.* (2018). doi:10.3390/jcm7080201
33. Bhowmick, R. & Gappa-Fahlenkamp, H. Cells and Culture Systems Used to Model the Small Airway Epithelium. *Lung* (2016). doi:10.1007/s00408-016-9875-2
34. Nakanishi, J., Takarada, T., Yamaguchi, K. & Maeda, M. Recent advances in cell micropatterning techniques for bioanalytical and biomedical sciences. *Analytical Sciences* (2008). doi:10.2116/analsci.24.67

35. Berthier, E., Young, E. W. K. & Beebe, D. Engineers are from PDMS-land, biologists are from polystyrenia. *Lab on a Chip* (2012). doi:10.1039/c2lc20982a

36. Halldorsson, S., Lucumi, E., Gómez-Sjöberg, R. & Fleming, R. M. T. Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices. *Biosensors and Bioelectronics* (2015). doi:10.1016/j.bios.2014.07.029

37. Sidhaye, V. K., Schweitzer, K. S., Caterina, M. J., Shimoda, L. & King, L. S. Shear stress regulates aquaporin-5 and airway epithelial barrier function. *Proc. Natl. Acad. Sci. U. S. A.* (2008). doi:10.1073/pnas.0712287105

38. Spassov, S. G., Kessler, C., Jost, R. & Schumann, S. Ventilation-like mechanical strain modulates the inflammatory response of BEAS2B epithelial cells. *Oxid. Med. Cell. Longev.* (2019). doi:10.1155/2019/2769761

39. Pezzulo, A. A. *et al.* The air-liquid interface and use of primary cell cultures are important to recapitulate the transcriptional profile of in vivo airway epithelia. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* (2011). doi:10.1152/ajplung.00256.2010

40. Karp, P. H. *et al.* An in vitro model of differentiated human airway epithelia. Methods for establishing primary cultures. *Methods Mol. Biol.* (2002). doi:10.1385/1-59259-185-X:115

41. Yamaya, M., Finkbeiner, W. E., Chun, S. Y. & Widdicombe, J. H. Differentiated structure and function of cultures from human tracheal epithelium. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* (1992). doi:10.1152/ajplung.1992.262.6.l713

42. Whitcutt, M. J., Adler, K. B. & Wu, R. A biphasic chamber system for maintaining polarity of differentiation of culture respiratory tract epithelial cells. *Vitr. Cell.*

Dev. Biol. (1988). doi:10.1007/BF02628493

43. Castellone, R. D., Leffler, N. R., Dong, L. & Yang, L. V. Inhibition of tumor cell migration and metastasis by the proton-sensing GPR4 receptor. *Cancer Lett.* (2011). doi:10.1016/j.canlet.2011.08.013

44. Kramer, N. *et al.* In vitro cell migration and invasion assays. *Mutation Research - Reviews in Mutation Research* (2013). doi:10.1016/j.mrrev.2012.08.001

45. Grainger, C. I., Greenwell, L. L., Lockley, D. J., Martin, G. P. & Forbes, B. Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. *Pharm. Res.* (2006). doi:10.1007/s11095-006-0255-0

46. Joint Annual Meeting of the Swiss Respiratory Society Swiss Society of Oto-Rhino-Laryngology, Head and Neck Surgery Swiss Paediatric Respiratory Society Swiss Society for Thoracic Surgery Interlaken, May 4–6, 2011. *Respiration* (2011). doi:10.1159/000327394

47. Trieu, D., Waddell, T. K. & McGuigan, A. P. A microfluidic device to apply shear stresses to polarizing ciliated airway epithelium using air flow. *Biomicrofluidics* (2014). doi:10.1063/1.4901930

48. Javaherian, S., O'Donnell, K. A. & McGuigan, A. P. A fast and accessible methodology for micro-patterning cells on standard culture substrates using parafilm™ inserts. *PLoS One* (2011). doi:10.1371/journal.pone.0020909

49. Nyegaard, S., Christensen, B. & Rasmussen, J. T. An optimized method for accurate quantification of cell migration using human small intestine cells. *Metab. Eng. Commun.* (2016). doi:10.1016/j.meteno.2016.03.002

50. Poon, J. C. H. *et al.* Design of biomimetic substrates for long-term maintenance of alveolar epithelial cells. *Biomater. Sci.* (2018). doi:10.1039/c7bm00647k

51. Wu, C. *et al.* Lift-off cell lithography for cell patterning with clean background. *Lab Chip* (2018). doi:10.1039/c8lc00726h

52. Rodriguez, L. G., Wu, X. & Guan, J. L. Wound-healing assay. *Methods Mol. Biol.* (2005). doi:10.1385/1-59259-860-9:023

53. Riahi, R., Yang, Y., Zhang, D. D. & Wong, P. K. Advances in wound-healing assays for probing collective cell migration. *Journal of Laboratory Automation* (2012). doi:10.1177/2211068211426550

54. Pijuan, J. *et al.* In vitro cell migration, invasion, and adhesion assays: From cell imaging to data analysis. *Front. Cell Dev. Biol.* (2019). doi:10.3389/fcell.2019.00107

55. Groß, A., Chernyakov, D., Gallwitz, L., Bornkessel, N. & Edemir, B. Deletion of von hippel–lindau interferes with hyper osmolality induced gene expression and induces an unfavorable gene expression pattern. *Cancers (Basel).* (2020). doi:10.3390/cancers12020420

56. Zordan, M. D., Mill, C. P., Riese, D. J. & Leary, J. F. A high throughput, interactive imaging, bright-field wound healing assay. *Cytom. Part A* (2011). doi:10.1002/cyto.a.21029

57. Kumar, P., Nagarajan, A. & Uchil, P. D. Analysis of cell viability by the lactate dehydrogenase assay. *Cold Spring Harb. Protoc.* (2018). doi:10.1101/pdb.prot095497

58. Tang, P., Xu, J., Oliveira, C. L., Li, Z. J. & Liu, S. A mechanistic kinetic description of lactate dehydrogenase elucidating cancer diagnosis and inhibitor evaluation. *J. Enzyme Inhib. Med. Chem.* (2017). doi:10.1080/14756366.2016.1275606

59. Wan, H. *et al.* Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 anmd 16HBE14o-. *Eur. Respir. J.* (2000). doi:10.1034/j.1399-3003.2000.01514.x

60. Martens, K., Hellings, P. W. & Steelant, B. Calu-3 epithelial cells exhibit different immune and epithelial barrier responses from freshly isolated primary nasal epithelial cells in vitro. *Clin. Transl. Allergy* (2018). doi:10.1186/s13601-018-0225-8

61. Denning, G. M. *et al.* Pseudomonas pyocyanin increases interleukin-8 expression by human airway epithelial cells. *Infect. Immun.* (1998). doi:10.1128/iai.66.12.5777-5784.1998

62. Dabaghi, M. *et al.* Microfluidic blood oxygenators with integrated hollow chambers for enhanced air exchange from all four sides. *J. Memb. Sci.* (2020). doi:10.1016/j.memsci.2019.117741

63. Ramirez, R. D. *et al.* Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. *Cancer Res.* (2004). doi:10.1158/0008-5472.CAN-04-3703

64. Moheimani, F. *et al.* Influenza A virus infection dysregulates the expression of microRNA-22 and its targets; CD147 and HDAC4, in epithelium of asthmatics.

Respir. Res. (2018). doi:10.1186/s12931-018-0851-7

65. Warner, S. M. B. *et al.* Transcription factor p63 regulates key genes and wound repair in human airway epithelial Basal cells. *Am. J. Respir. Cell Mol. Biol.* (2013). doi:10.1165/rcmb.2012-0447OC

66. Da Paula, A. C. *et al.* Characterization of novel airway submucosal gland cell models for cystic fibrosis studies. *Cell. Physiol. Biochem.* (2005). doi:10.1159/000087235

67. Joo, N. S., Lee, D. J., Winges, K. M., Rustagi, A. & Wine, J. J. Regulation of antiprotease and antimicrobial protein secretion by airway submucosal gland serous cells. *J. Biol. Chem.* (2004). doi:10.1074/jbc.M407077200

68. Dubin, R. F., Robinson, S. K. & Widdicombe, J. H. Secretion of lactoferrin and lysozyme by cultures of human airway epithelium. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* (2004). doi:10.1152/ajplung.00326.2003

69. Zhang, Y., Reenstra, W. W. & Chidekel, A. Antibacterial activity of apical surface fluid from the human airway cell line Calu-3: Pharmacologic alteration by corticosteroids and β2-agonists. *Am. J. Respir. Cell Mol. Biol.* (2001). doi:10.1165/ajrcmb.25.2.4211

70. Zhu, Y., Chidekel, A. & Shaffer, T. H. Cultured Human Airway Epithelial Cells (Calu-3): A Model of Human Respiratory Function, Structure, and Inflammatory Responses. *Crit. Care Res. Pract.* (2010). doi:10.1155/2010/394578

71. Winkler, T. E., Feil, M., Stronkman, E. F. G. J., Matthiesen, I. & Herland, A. Low-cost microphysiological systems: Feasibility study of a tape-based barrier-on-chip

for small intestine modeling. *Lab Chip* (2020). doi:10.1039/d0lc00009d

72. Strober, W. Trypan blue exclusion test of cell viability. *Curr. Protoc. Immunol.* (2001). doi:10.1002/0471142735.ima03bs21

73. Chawla, A. S. Toxicity evaluation of a novel filler free silicone rubber biomaterial by cell culture techniques. *J. Biomed. Mater. Res.* (1982). doi:10.1002/jbm.820160415

74. MacDougall, J. D. B. Toxicity studies on silicone rubber and other substances [21]. *Nature* (1953). doi:10.1038/172124c0

75. Mojsiewicz-Pienkowska, K., Jamrógiewicz, M., Szymkowska, K. & Krenczkowska, D. Direct human contact with siloxanes (silicones) - safety or risk part 1. Characteristics of siloxanes (silicones). *Frontiers in Pharmacology* (2016). doi:10.3389/fphar.2016.00132

76. Krenczkowska, D. *et al.* The consequences of overcoming the human skin barrier by siloxanes (silicones) Part 1. Penetration and permeation depth study of cyclic methyl siloxanes. *Chemosphere* (2019). doi:10.1016/j.chemosphere.2018.09.154

77. Jonkman, J. E. N. *et al.* An introduction to the wound healing assay using live-cell microscopy. *Cell Adhesion and Migration* (2014). doi:10.4161/cam.36224

78. Stamm, A. *et al.* In vitro wound healing assays - State of the art. *BioNanoMaterials* (2016). doi:10.1515/bnm-2016-0002

79. Sven, K. & Josipa, F. Interstitial hydrostatic pressure: a manual for students. *Adv. Physiol. Educ.* (2007). doi:10.1152/advan.00084.2006

80. Tarran, R. *et al.* Normal and cystic fibrosis airway surface liquid homeostasis: The

effects of phasic shear stress and viral infections. *J. Biol. Chem.* (2005). doi:10.1074/jbc.M505832200

81. Tsukihara, S. *et al.* Interleukin-1beta-induced Expression of IL-6 and Production of Human Chorionic Gonadotropin in Human Trophoblast Cells via Nuclear Factor-kappaB Activation. *Am. J. Reprod. Immunol.* (2004). doi:10.1111/j.1600-0897.2004.00209.x

82. Van Den Berg, A. *et al.* Interleukin-17 induces hyperresponsive interleukin-8 and interleukin-6 production to tumor necrosis factor-α in structural lung cells. *Am. J. Respir. Cell Mol. Biol.* (2005). doi:10.1165/rcmb.2005-0022OC

83. Herseth, J. I., Volden, V., Schwarze, P. E., Låg, M. & Refsnes, M. IL-1beta differently involved in IL-8 and FGF-2 release in crystalline silica-treated lung cell co-cultures. *Part. Fibre Toxicol.* (2008). doi:10.1186/1743-8977-5-16

84. Kim, G.-Y. *et al.* Proinflammatory Cytokine IL-1β Stimulates IL-8 Synthesis in Mast Cells via a Leukotriene B 4 Receptor 2-Linked Pathway, Contributing to Angiogenesis . *J. Immunol.* (2010). doi:10.4049/jimmunol.0901735

85. Jonczyk, R. *et al.* Living Cell Microarrays: An Overview of Concepts. *Microarrays* (2016). doi:10.3390/microarrays5020011

86. Ziauddin, J. & Sabatini, D. M. Microarrays of cells expressing defined cDNAs. *Nature* (2001). doi:10.1038/35075114