Advanced *in vitro* systems available for respiratory toxicology

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1 Introduction to *In Vitro* Inhalation Testing

Increasing concerns in respect to the transferability of data from animal studies to real human health effects further supported by established legal frameworks in many juridical areas have pushed the development of advanced *in vitro* models (Stucki *et al.*, 2022). Such development of complex *in vitro* cell models based on human cells can clearly contribute to potentially replace ethically and often scientifically debatable *in vivo* studies (Clippinger *et al.*, 2021; Stucki *et al.*, 2022). Attempts to fill the existing gap between *in vivo* and *in vitro* data have received considerable attention.

This chapter focuses on available *in vitro* cellular models mimicking the various anatomical parts of the respiratory tract which are currently available for studying the biological effects elicited by inhaled chemicals, (nano)materials or other agents. The advantages and disadvantages of monoculture systems and more complex models such as co-cultures and organ-on-a-chip platforms will be explained and discussed. Special focus will be given on the need to culture cells from the respiratory tract at the air-liquid-interphase (ALI) (Lacroix *et al.*, 2018) and the added value of complexity (Marescotti *et al.*, 2019). The question of quality control of work in an *in vitro* laboratory, which is covered by an OECD endorsed approach known as "Good *in vitro* Method Practice" (GIVIMP) will be explained in a specific topic (OECD, 2018). Future perspectives of such *in vitro* cellular models in respiratory toxicology will be presented.

In the last decade, a focus in developing complex *in vitro* models representing different anatomical regions of the pulmonary system from the nasal epithelial barrier all the way to the alveolar region can be observed (Klein *et al.*, 2013; Tosoni *et al.*, 2016; Walls *et al.*, 2024). Such models can be based on differentiated primary cells, induced pluripotent stem cells (iPSCs) or cell lines. The cells can be cultured in the form of a single cell type or as co-cultures cultured on porous membranes, (bio-)engineered scaffolds, spheroid or organoid models and microfluidic systems (**Fig. 1**). Such models hold the promise for a more thorough and relevant resemblance of the *in vivo* situation allowing the characterization of potentially detrimental effects of inhalable chemicals or materials. All such *in vitro*

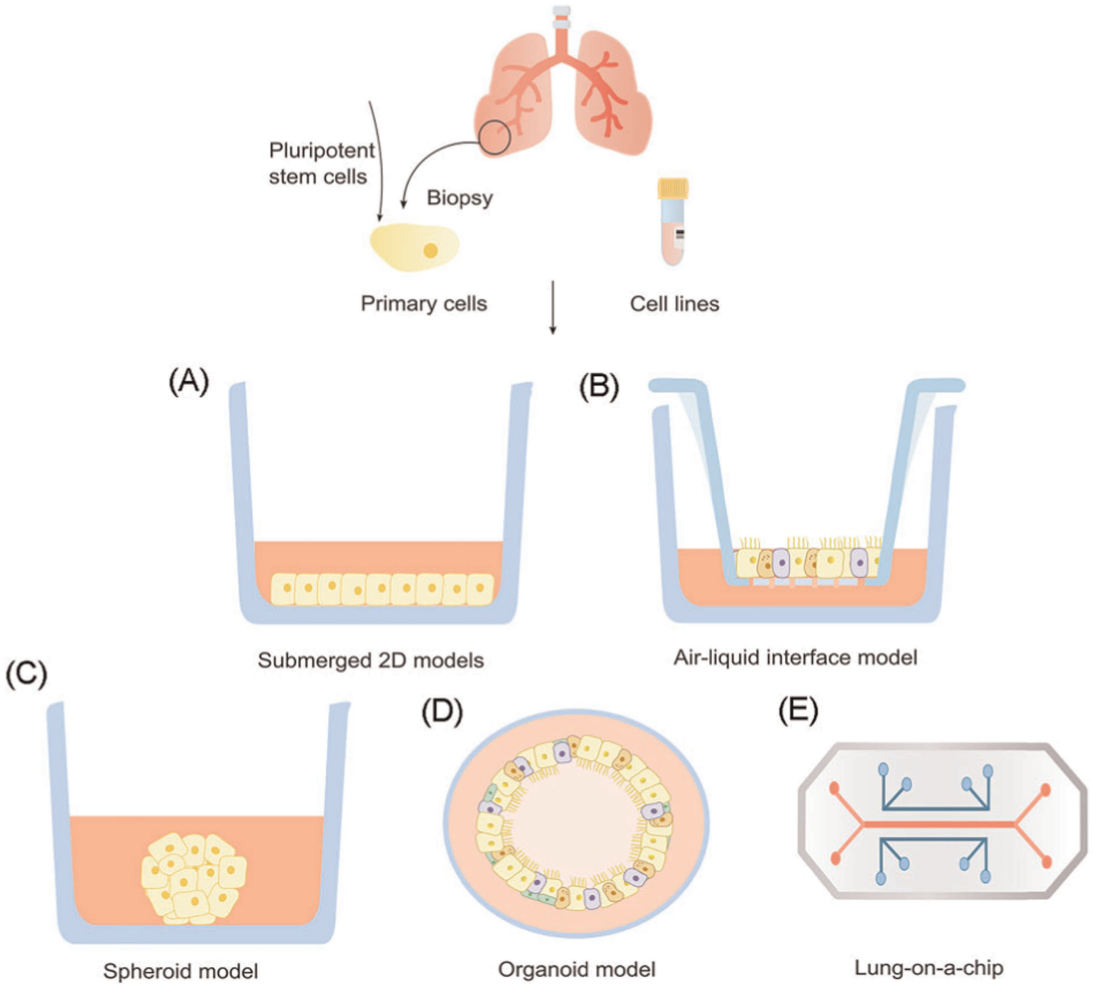

Fig. 1 Overview of the most commonly used *in vitro* cellular models in pneumonia research. (A) While still broadly used, the standard submerged model has a lower translational value compared to the more advanced cellular models. (B) Air-liquid interphase models allow for cell differentiation by exposing cell monolayers to air resulting in a pseudostratified epithelium and/or exposure to aerosols and gases. (C) Spheroid models have a three-dimensional structure but lack self-renewing capacity. (D) Organoids are more complex three-dimensional cell structures with an organ-like architecture, including tissue lumen and self-renewal. (E) Lung-on-a-chip devices contain multiple chambers where cells are seeded and exposed to a dynamic microenvironment, including a continuous air and medium flow, and fluid shear stress. From Mahieu *et al.* (2024). *FEMS Microbiology Reviews*, 48, fuae007.

methods plus *in silico* approaches are collectively addressed as New Approach Methodologies (NAMS) and get an increasing attention from regulators in how they are fit-for-purpose from a regulatory perspective (Stucki *et al.*, 2022; van der Zalm *et al.*, 2022).

Endpoints studied in such systems can range from inflammation (Refsnes *et al.*, 2023), oxidative stress (Klein *et al.*, 2013), sensitization (Chary *et al.*, 2019; Hargitai *et al.*, 2024), cytochrome induction (Refsnes *et al.*, 2023), genotoxicity (Azzurra Cammassa *et al.* 2022), agonistic effects of drug candidates (Barbot *et al.*, 2024), to cell hyperplasia as a model for precancerous lesions induced by exposure to BaP (Aufderheide *et al.*, 2016).

Another important step for the improvement of *in vitro* models in respiratory toxicology was the development of exposure systems, which allow the direct exposure to aerosols containing the inhalable material, thus closely mimicking native respiratory exposure. This is especially true for inhalable particles where the direct exposure of cells to the aerosol is of utmost importance as the toxicity profiles can be considerably altered during conventional submerged exposure due to the formation of a protein corona on the surface of the particulate matter (Lynch *et al.*, 2007, 2013).

The aim of the current chapter is to describe the state-of-the-art of available *in vitro* models for studying the toxicity of inhalable toxicants on the pulmonary system. Advantages and limitations displayed by the current *in vitro* models will be systematically discussed starting from basic submerged monocultures and culminating with state-of the-art organ-on-a-chip (OoC) platforms.

However, only very limited validation studies have been carried out to date. This means comparison of data from different methods is difficult. This is despite inhalation toxicology being a priority area for many governmental organizations. There is a clear need for efforts to produce *in vitro* models ready to serve as a tool for regulators.

2 Submerged Versus ALI Exposure of *in Vitro* Models for the Respiratory Tract

A key element of any *in vitro* model is to reproduce the function and organization of the native tissue it represents, which in the case of the *in vitro* models described in this chapter is the epithelium of the respiratory tract, either in a healthy or in a diseased state.

Submerged cell culture techniques have historically been most widely used in *in vitro* studies and models representing practically a very wide variety of tissues, the value of submerged culture of cells from the respiratory tract for inhalation studies has increasingly been questioned (Lacroix *et al.*, 2018). This has two main reasons: 1) cells from the respiratory tract grow *in vivo* at the air-liquid interphase and not within a tissue such as e.g., liver cells etc. and it has been shown that cell properties change when cells are exposed at the ALI (Marescotti *et al.*, 2019). 2) Especially for the study of (nano)particles the fact that upon exposure to proteins in cell culture medium containing foetal bovine serum (FBS) immediately a protein corona forms (Lynch *et al.*, 2007, 2013), which changes the properties of the particles and of course does not happen on the surface of particles while in the airstream.

As a result of these two widely accepted points, acceptance of manuscripts presenting data from submerged cultured cells or complex barriers from the respiratory tract, has become increasingly difficult in most journals. However, due to the simplicity of the method and procedures, relevant experiments are still performed and published (van den Brule *et al.*, 2022).

In vitro ALI cell culture models have been increasingly used in recent years to assess endpoints relevant for inhalation toxicology for the reasons mentioned above (Lacroix *et al.*, 2018; Upadhyay and Palmberg, 2018). They practically have completely replaced classic submerged *in vitro* models when it comes to inhalation toxicology.

A recent study comparing submerged versus ALI exposure of A549 cells reported increased susceptibility of the epithelial barrier following exposure to several different engineered NPs at relevant occupational concentrations. Therefore, it was concluded that ALI exposure is the most suitable choice for hazard assessment of such materials (Bessa *et al.*, 2021). Culture at ALI conditions change the properties of the test system as for example increased trans epithelial electrical resistance (TEER) in adeno-carcinoma derived NCI-H441 lung cells was observed (Lochbaum *et al.*, 2020). ALI exposure to carbon nanotube exposure resulted in the increased expression of proteins connected with oxidative stress and these changes were not observed under submerged conditions (Hilton *et al.*, 2019).

Such ALI models are also increasingly replacing animal experiments especially for modes of action such as respiratory sensitization where anyhow no animal-based models exist (Chary *et al.*, 2018, 2019; Roper *et al.*, 2022).

ALI models have shown to be able to represent a complementary and often very useful alternative option to *in vivo* experiments. ALI culture induces cell differentiation; however, only very limited validation studies have been carried out to date. This means comparison of data from different methods is still difficult. This is despite the fact inhalation toxicology being a priority area for many governmental organizations. The question of variability and the sources of variability when using *in vitro* models at the ALI have been explored in a key publication dissecting in detail all steps of producing an ALI culture using a cause-effect analysis (Petersen *et al.*, 2021).

Cell culture at the ALI is not possible nor it is physiologically relevant for all kind of cells, however, various cell types (most notably epithelial and immune cells) from the respiratory tract (Burla *et al.*, 2023; Chary *et al.*, 2019; Klein *et al.*, 2011; Licciardello *et al.*, 2023) have been shown to be relative easily cultured at the ALI. When establishing a new cell type or complex model at the ALI it is advised to carefully characterize the properties of the cells under these conditions. Examples are the

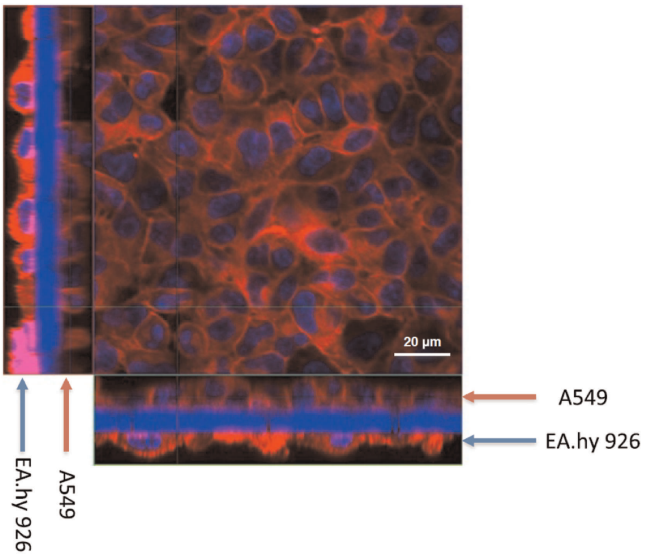

Fig. 2 Z-stack image series to evaluate the distribution of A549 and EA.hy 926 cells on opposite sides of a transwell insert. The cells form a closed monolayer on both sides of the 1 µm transwell membrane. Cellular membranes are stained in red (cell mask deep red dye), nuclei in blue (DAPI). X–y projection with the respective side views (Magnification: 630x). From Klein *et al.* (2013).

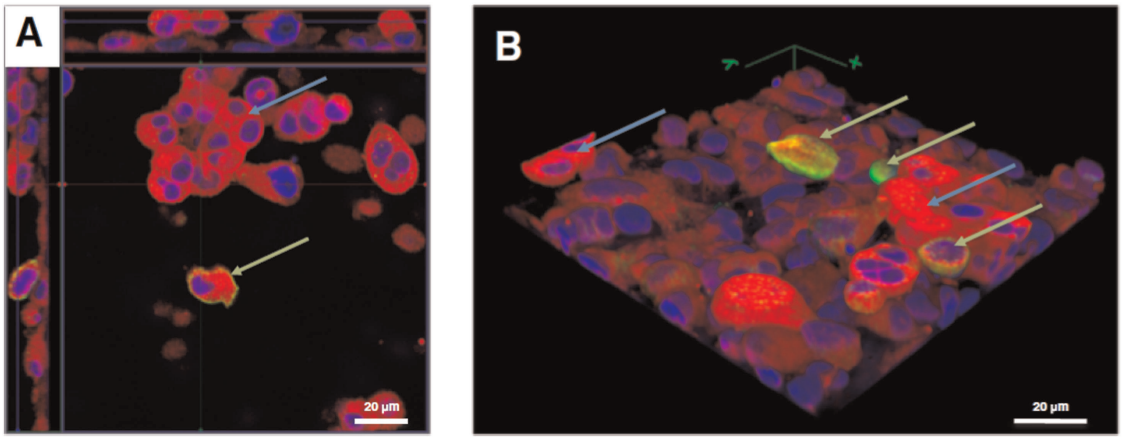

Fig. 3 Z-stack image series to analyse the distribution of THP-1 macrophages and HMC-1 in the tetraculture system present in the apical compartment of the insert. The distribution of A549, differentiated THP-1, HMC-1 and EA.hy 926 cells in the tetraculture was analysed via CLSM. Cellular membranes are stained with cell mask deep red dye (red) and nuclei are stained with DAPI (blue); Macrophage-like cells are counterstained with an anti-CD11b-antibody. A: X–y projection with the respective side views. B: 3D reconstruction of the tetraculture based on the results of the z-stack from A. THP-1 (green arrows) and HMC-1 (blue arrows) cells are found on top of the epithelial cells. EA.hy 926 cells were not considered in the 3D reconstruction. From Klein *et al.* (2013).

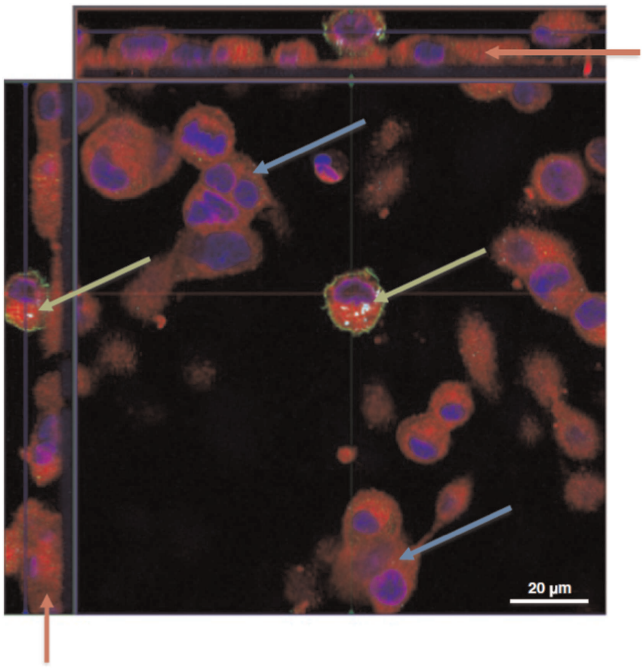

Fig. 4 Z-stack image series to analyse the phagocytic activity of THP-1 macrophage-like cells in the tetraculture present in the apical compartment of the system. Tetracultures of A549, differentiated THP-1, HMC-1 and EA.hy 926 were exposed to cell culture medium containing 10 mg/L of 50 nm SiO2-Rhodamine particles for 24 h. SiO2-Rhodamine particles distribution was analysed via CLSM. Cellular membranes are stained with cell mask deep red dye (red) and nuclei are stained with DAPI (blue). Macrophage cells are counterstained with an anti-CD11b-antibody (green). Fluorescence from ingested SiO2-Rhodamine particles was detected in differentiated THP-1 cells situated on top of the A549 cells (green arrows) but not in A549 (red arrows) or HMC-1 (blue arrows). The image shows an x–y projection with the respective side views. From Klein *et al.* (2013).

confluency of the cells (**Fig. 2**), presence and maturity of macrophages (**Fig. 3**), or the phagocytic activity of macrophages (**Fig. 4**). While it is true that assessing the general cell viability will give some information on the status of cells more specific assays are advisable to be performed in this phase.

Exposure of bronchial cells (HBEC3-KT) to real world traffic-derived particulate matter showed that the toxicity of organic particles and mineral particles was different between the investigated samples from different tunnels. There were also differences observed for the induction of CYP enzymes and inflammatory markers such as interleukin-6 (IL-6) (Refsnes *et al.*, 2023).

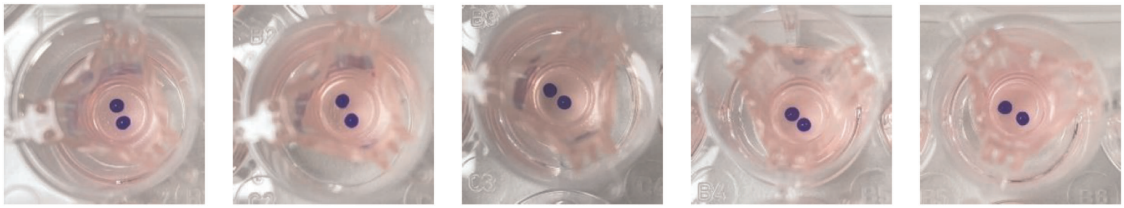

Fig. 5 Surfactant production and ALI maintenance following exposure of the *in vitro* model evaluated using the DMP/O droplet test. (a) unexposed (control) *in vitro* model; (b) 250 µL and (c) 500 µL H2O:DPBS= 1:1 (v/v) exposed, respectively; (d) 250 µL and (e) 500 µL DMSO:DPBS= 1:1 (v/v) exposed, respectively. All inserts were maintained for 24 h post-exposure and showed comparable DMP/O droplet diameters. From Burla *et al.* (2023).

In the alveolar region of the lung cells are covered by a thin layer of pulmonary surfactant containing phospholipids and several specific proteins. The presence of these proteins such as surfactant protein A-, B-, or C in A549 can be shown by dedicated immunostaining (Klein *et al.*, 2011). However, such staining only shows the intracellular presence of the surfactant proteins but not their secretion to the surface, which is necessary for cells to grow at the ALI and for a functional alveolar barrier. The presence of the surfactant on top of the epithelial layer after 24-hour culture at the ALI can be shown using a specific dye (Burla *et al.*, 2023; Klein *et al.*, 2013; Licciardello *et al.*, 2023) originally developed to measure surface tension in lungs *ex vivo* (Schürch *et al.*, 1976) (**Fig. 5**). The fact that A549 cells secret surfactant thereby being protected from desiccation is underlined by the fact that A549 cells have been cultured at the ALI for periods of up to 14 days (Wu *et al.*, 2017). Surfactant secretion is not restricted to A549 cells only but has also been described for hAELVi (Kletting *et al.*, 2018).

3 Dosimetry

Dosimetry of *in vitro* experiments and the relevance of the doses applied is of immense importance and comparison to concentrations in ambient air to which humans are exposed in real life is of utmost importance. In an *in vitro* study using A549 cells exposed to petroleum substances results showed consistency between the experimentally used concentrations and those that induce adverse effects *in vivo* (Verstraelen *et al.*, 2021). Careful evaluation of the delivered dose to an *in vitro* model must be done considering delivery efficacy of the exposure equipment and the plate size used as factors strongly influencing the applied dose. While human exposure is usually considered for an 8-hour working day and often a lifelong worker exposure, *in vitro* exposure is often done in the form of a single short term bolus application at unrealistic high concentrations (similar to *in vivo* acute toxicity testing in rats) and not many *in vitro* repeat-dose studies were published. Many studies only report the applied dose of particles but not the dose really delivered to the cells in the insert. Studies reporting concentrations of particles such as diesel exhaust or similar in the µg/cm^2 or even mg/cm^2 are of no relevance as exposure of tenths to hundreds of ng/cm^2 still represent a 24-hour worst case scenario (Klein *et al.*, 2017).

To characterize aerosols, gas or particle analyzers can be installed inline (Oeder *et al.*, 2015). For the measurement of the deposited mass, Quartz Crystal Microbalances (QCMs) can be used parallel or after the exposure of cell culture inserts (Mülhopt *et al.*, 2009). In cases where the deposited mass cannot be analysed chemical analysis may be an alternative and has successfully been applied to diesel exhaust particles (Klein *et al.*, 2017).

4 Monoculture Systems

Although classical monocultures based on cells originating from specific anatomical areas of the respiratory tract do not reflect the complexity of the whole respiratory system, their usefulness may have some merits in deciphering key aspects underlying physiological and pathophysiological processes in certain circumstances (van den Brule *et al.*, 2022). However, the simplicity of such models already mentioned above needs consideration.

5 Complex Multicellular Systems

Complex cellular models cultivated on bio-engineered scaffolds or inserts of various pore sizes allowing a 3D orientation (scaffolds) or two-sided and ALI cultures (inserts) have emerged and thereby opened completely new possibilities (Chary *et al.*, 2019; Klein *et al.*, 2013, 2017; Licciardello *et al.*, 2023; Rothen-Rutishauser *et al.*, 2008). Such models allow to mimic the extracellular environment and the interaction of multiple cell types in close vicinity to each other. One common feature for the establishment of complex multicell-type co-culture models is the careful characterization initially needed and seen in the key references for most of such models (Chary *et al.*, 2019; Klein *et al.*, 2013; Kletting *et al.*, 2018; Licciardello *et al.*, 2023).

It must be noted that the establishment of co-cultures can be a laborious and tricky task, as often different type of cells needs their own type of culture medium and supplements and optimum ratios or adaptations of cells to cell media may be needed

(Chary *et al.*, 2022; Klein *et al.*, 2013; Licciardello *et al.*, 2023). Based on these considerations, co-cultures of lung epithelial cells with other cell types including immune cells (such as differentiated macrophages, mast cells and dendritic cells), fibroblasts and endothelial cells have been promoted as tools to study pathophysiological mechanisms upon exposure *in vitro* (Alfaro-Moreno *et al.*, 2008; Chary *et al.*, 2019; Klein *et al.*, 2011, 2013, 2017; Lehmann *et al.*, 2011).

Cells can have an orientation mimicking the *in vivo* situation as was shown by the localization of macrophages and dendritic cells on the two sides of an alveolar barrier (**Fig. 6**).

Co-cultivation of cells from various areas of the pulmonary tract representing the epithelial or endothelial barrier, or which represent the immune system has allowed to study proper cell-to-cell communication (Burla *et al.*, 2023; Klein *et al.*, 2013) (**Fig. 7**), which underlies complex processes such as inflammation (Klein *et al.*, 2011) and sensitization (Chary *et al.*, 2018). It is this cell-to-cell communication that is a central in repair of epithelial injury in the pulmonary tract and which in part has been studied *in vitro* (Lucchini *et al.*, 2021).

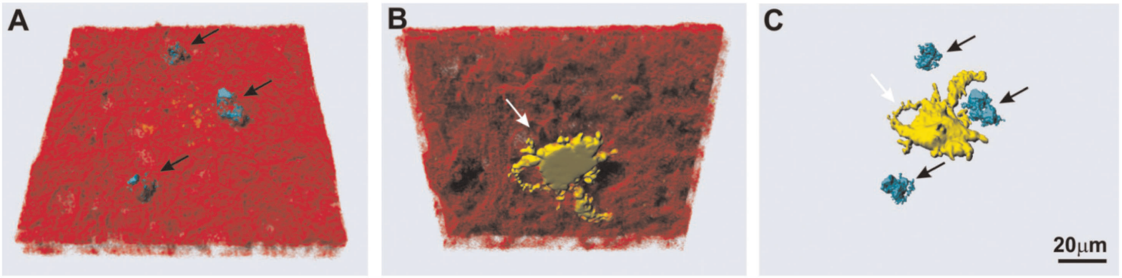

Fig. 6 LSM images of the triple cell co-culture model. Epithelial cells (red, volume rendering), monocyte derived macrophages (light blue, surface rendering; black arrows) and monocyte derived dendritic cells (yellow, surface rendering; white arrow) are shown. The same data set is shown from top (A), from bottom (B) and without epithelial cells from top (C). From Rothen-Rutishauser *et al.* (2008).

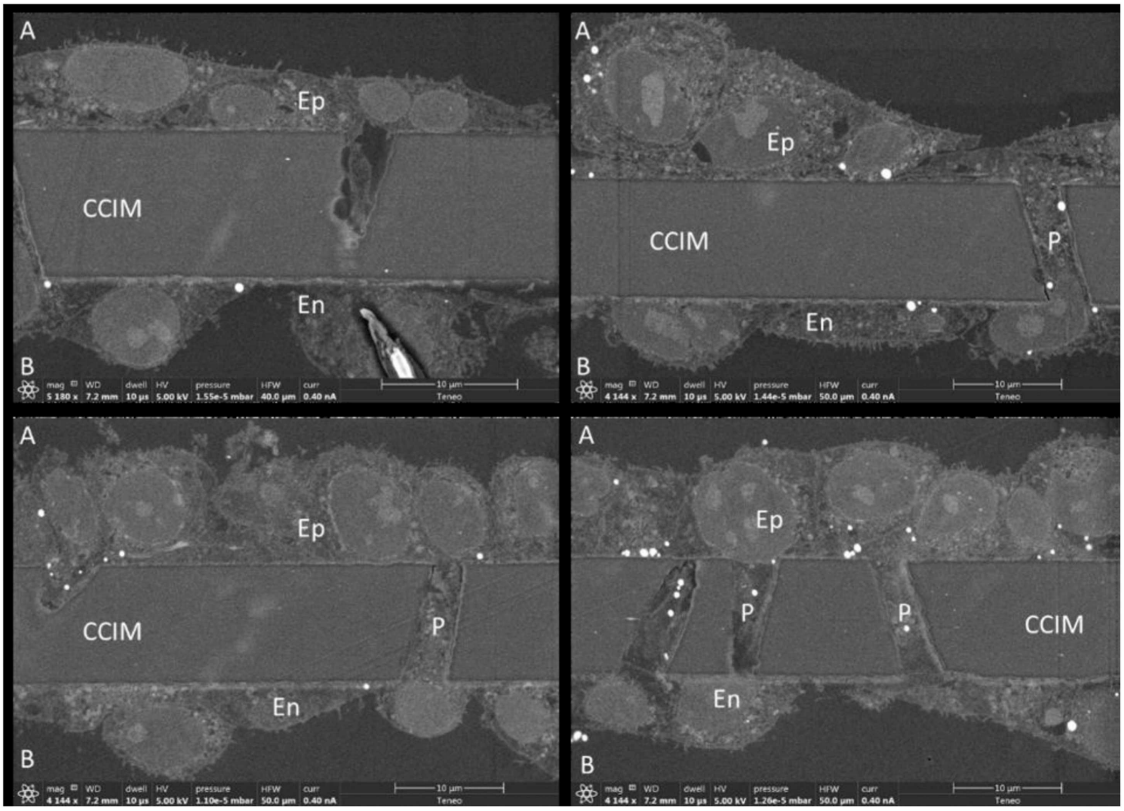

Fig. 7 Electron microscopy (EM) images illustrating the *in vitro* model. A – apical compartment; B – basolateral compartment; CCIM – cell culture insert membrane; En – endothelial cells (EA.hy926) monolayer; Ep – epithelial cells (A549) monolayer; P – cell culture insert membrane pore (Scale bar 10 µm). From Burla *et al.* (2023).

While usually several cell lines or different primary cells such as in the commercial models are co-cultured, also combinations of cell lines such as A549 in combination with primary human immune cells have been used to study for example interaction of surfactant protein D with plant derived allergens (Schleh *et al.*, 2012).

Despite that 3D models have an epithelial barrier on top of a membrane with varying pore size effects of exposure can be observed in cells of the lower compartment. In a tetra-culture representing the alveolar barrier consisting of A549, EA.hy 926 endothelial cells, HMC-1 mast cells and PMA differentiated THP-1 macrophages exposed to diesel exhaust particles at the ALI only on the apical side, 4 h post exposure nuclear translocation of the NRF2 receptor was reported at doses ranging from 80 ng/cm^2 to 240 ng/cm^2 (Klein *et al.*, 2017). Similar effects were observed in a model for the bronchial barrier, where underlying fibroblasts separated from the bronchial cells by a membrane showed increased accumulation of hydrogen peroxide, activation of NRF2, and induction of oxidative stress-responsive genes (Faber *et al.*, 2020).

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6 Different Well Formats and Downscaling of *in Vitro* Models

Downscaling *in vitro* models from the 6-well format, the plate format often used for method development and in academic settings to smaller well formats that are used in various industries to increase throughput and allow to test more chemicals faster and cheaper is a complex task that asks for a dedicated approach. Such downscaling needs thorough characterization very similar in respect to time and resources to the efforts needed for establishing the original model.

A successful example of such a dedicated effort is the downscaling of a 6-well format model developed to identify respiratory sensitizers (Chary *et al.*, 2019). Exposure of the final version of the 24-well model and the observed identical results upon exposure to the test chemicals proved the functionality of the downscaled *in vitro* model (Burla *et al.*, 2023).

Downscaled *in vitro* models present a series of advantages for future use: (1) decreased use of cell culture consumables such as flasks, tubes, and other plastic ware and reagents such, cell culture media, growth factors or coatings, as well potentially expensive/rare test substances, etc.; (2) increased throughput for the number of test materials; (3) the possibility to combine *in vitro* models of the lung with *in vitro* models representing other organs in microfluidics for systemic studies (Burla *et al.*, 2023).

7 Cell Types Used to Establish *in Vitro* Models for the Respiratory System

For the establishment of *in vitro* models for the respiratory tract primary cells isolated from patient tissue of established and commercially available cell lines can be used (**Fig. 8**).

Cell lines are the backbone and workhorse of *in vitro* technology, and this is also true for models resembling the human pulmonary system (**Table 1**). Many cell lines originate from human primary tumours. Advantages of cell lines are the storing possibility as frozen stocks which makes them available at any timepoint for experiments requesting minimum planning. Cell lines are usually well characterized in respect to eventual presence of virus or their metabolic activity (Courcot *et al.*, 2012; Oesch *et al.*, 2019). They can be produced in large numbers at plannable timepoints which makes the experimental planning relatively easy and foreseeable. More recently and with the development of advanced molecular tools immortalization and transfection techniques have been applied to primary cells to establish stable cell lines (Kemp *et al.*, 2008; Kletting *et al.*, 2018; Sengupta *et al.*, 2023; Walters *et al.*, 2013). Similar to cell lines derived from tumours their intrinsic properties such as gene expression patterns may be altered considerably by this process. Nevertheless, immortalized human alveolar epithelial cells showed the expected changed pattern of several relevant enzymes and morphological changes (Kemp *et al.*, 2008).

In recent years the origin of the cells, meaning whether the donor was male, or female has attracted some interest from the scientific community (Gutleb and Gutleb, 2023).

When cancer cell lines are used, which are often the basic tool in establishing *in vitro* models, one must be aware that several important aspects of cell physiology such as apoptotic processes may be quite different in these cell lines from normal cells.

8 Primary Cells

Primary cultures of normal tissue can be used to study normal physiology of different cell types. Primary cell cultures are often used in comparison with cancer cell lines to study cancer specific traits. One advantage of primary cells is that the differences within a population can be confirmed by testing cell cultures from numerous individuals.

One feature of using primary cells is the fact that the differences between the donors will become visible, which can be seen as an advantage or disadvantage dependent on the question investigated. When using primary nose epithelial cells to study donor variability using TEER as the readout large differences between donors but also for the variability within tissues from the same donor were observed. TEER values varied by a factor of ca. four and the variation of tissues from the same donor was even higher (Tosoni *et al.*, 2016). In a study evaluating aged gasoline exhaust particles cell models based on primary cells from cystic fibrosis

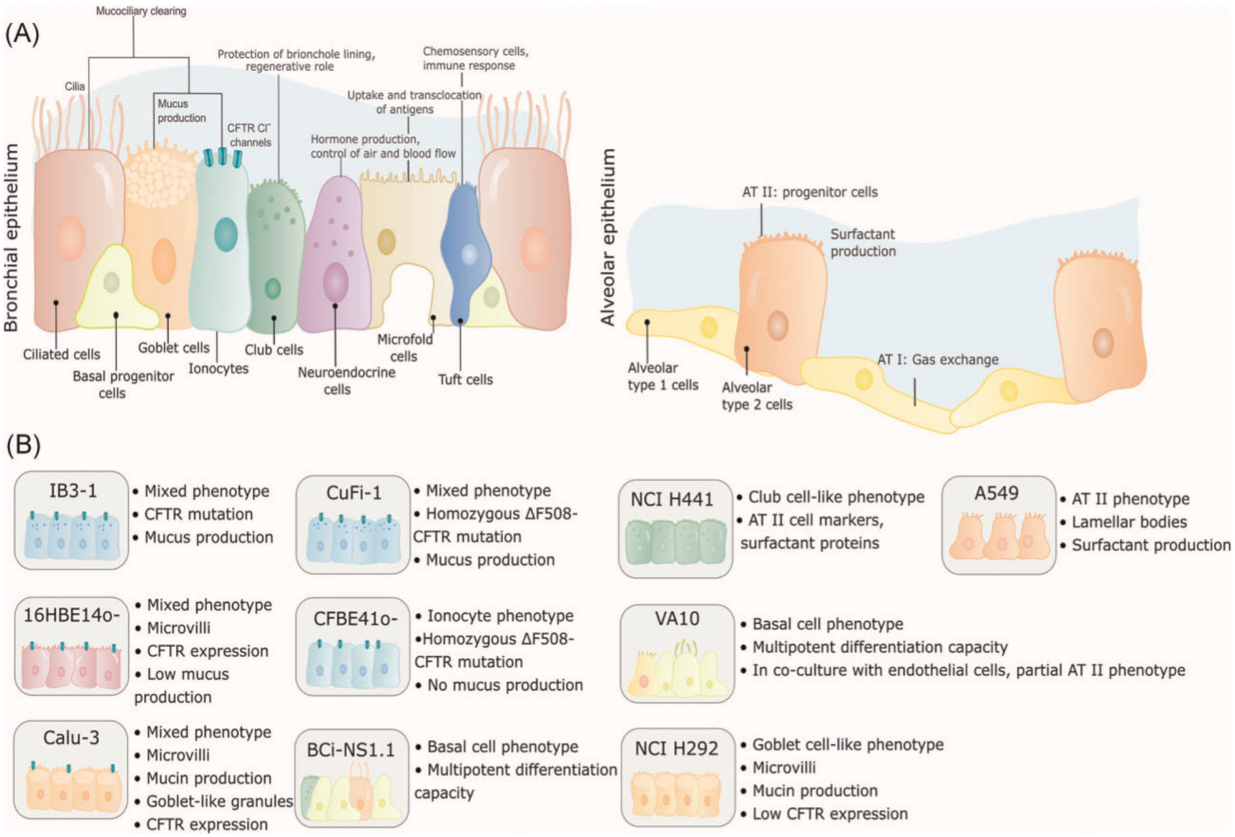

Fig. 8 (A) Main cells of the differentiated bronchial and alveolar epithelium. The respiratory epithelium is characterized by a multitude of cell subsets, including ciliated cells and serous goblet cells, club cells and basal progenitor cells. More rare cell types include the ionocytes, microfold cells, neuroendocrine cells, and tuft cells. The alveolar epithelium mainly consists of two cell types, the alveolar type I (ATI) and type II cells (ATII). (B) Cell lines used to establish complex bacterial infection models and some key characteristics are depicted. CFTR = cystic fibrosis transmembrane conductance regulator. From Mahieu *et al.* (2024).

showed stronger responses than models from healthy donors and a bronchial cell line (BEAS-2B) (Künzi *et al.*, 2015). This shows that such models may give valuable information for vulnerable parts of the population.

It must be noted that there are also models for healthy and diseased tissues existing that can be purchased as complex models ready to be used from commercial providers. These models can include ciliated cells, goblet cells that secret a functional mucus and basal cells (**Fig. 9**) and model upper airways (MucilAir™, Epithelix, Geneve, CH; EpiNasal and EpiAirway, MatTek, Ashland, US), lower airways (SmallAir™, Epithelix; NewCells, Newcastle, UK), and can include fibroblasts (EpiAirway, MatTek; MucilAir™-HF, SmallAir™-HF, Epithelix) or represent the alveolar barrier incorporating ATI, ATII and endothelial cells (AlveolAir™, Epithelix; EpiAlveolar™, MatTek) (Balogh Sivars *et al.*, 2018). These models have a good shelf-life and can be shipped almost globally and are reasonably standardized. To tackle potential inter-individual variability pool-of-donors versions are available (Epithelix). Some of the models also exist based on cells from diseased donors and can represent asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, lung fibrosis and rhinitis (Epithelix, MatTek) (Barbot *et al.*, 2024). The ImmuLUNG cell model is representing the alveolar region and consists of alveolar epithelial cells in combination with alveolar-like macrophages. The model has been utilized for the detection of irritation and sensitization markers such as cell surface markers, cytokines and chemokines (Hutter *et al.*, 2023).

One example of applying these models is a study in which micro- and nanoplastic particles from different materials, were tested in healthy MucilAir tissue (Epithelix). Some of the different materials tested induced secretion of inflammatory markers such as IL-6 showing the applicability of such models for difficult to test materials (Donkers *et al.*, 2022). Another study used cell-line based models and SmallAir™ and MucilAir™ in combination with a human lung microfluidic 3D system PhysioMimix™ (CNBio Innovations, Welwyn Garden, UK) in an attempt to develop models for accelerated drug testing. The results show a high relevance of such models for drug and toxicant testing but also for the hazard assessment of materials in an occupational setting (Phan *et al.*, 2023).

Another important aspect which is not much explored is the fact that at least for primary bronchial cells functional differences were observed dependent on the medium used to culture human bronchial epithelial cells (Leung *et al.*, 2020).

Table 1 Common human cell lines used to establish *in vitro* models for the pulmonary system

Name	*Origin*	*Sex*	*Described for*	*Endpoints*	*Reference*
Pulmonary cells					
A549	adeno-carcinoma	m	mono-, co-culture	inflammation, cytotoxicity, oxidative stress, resp. sensitization	Chary *et al.*, 2019; Klein *et al.*, 2013
NCI-H441	papillary adeno-carcinoma	m	monoculture	cytotoxicity	Uboldi *et al.*, 2009
hAELVi	alveolar epithelial lentivirus immortalized	m	mono-, co-culture	inflammation, oxidative stress	Kletting *et al.*, 2018
Arlo	monoclonal development of hAELVI	m	mono-, co-culture	drug permeability, viral infections	Carius *et al.*, 2023
HBEC3-KT	hTERT-immortalized epithelial cell	f	monoculture	inflammation	Werner *et al.*, 2015
Calu-3	bronchial carcinoma	m	monoculture	transport studies	Grainger *et al.*, 2006
16HBE14o	bronchial epithelium	?	monoculture	oxidative stress and inflammation	Hussain *et al.*, 2009
BEAS-2B	bronchial epithelium	m	monoculture	uptake of particles	Penn *et al.*, 2005
BET-1A	bronchial epithelium	m	monoculture	inflammation	Abe *et al.*, 2000
NCI-H292	mucoepidermoid pulmonary carcinoma	m	monoculture	cytotoxicity	Phillips *et al.*, 2005
NHBE	primary bronchial epithelial	m/f	Co-culture	apoptosis	Gray *et al.*, 2007
Endothelial cells					
HULEC-5a	immortalized pulmonary endothelium	m	co-culture	inflammation	Licciardello *et al.*, 2023
HUVEC	hTERT immortalized endothelial cells	f	co-culture	drug development	Cao *et al.*, 2017
HMVEC	primary lung microvascular endothelial cells	–	co-culture	drug development	An *et al.*, 2022
EA.hy 926	Somatic hybrid of umbilical vein cells and A549	m	co-culture	Inflammation, resp. sensitization	Chary *et al.*, 2019; Klein *et al.*, 2013
Immune cells					
THP-1	acute monocytic leukaemia	m	monoculture, co-culture	Inflammation, cytotoxicity, resp. sensitization	Alfaro-Moreno *et al.*, 2008; Chary *et al.*, 2019; Klein *et al.*, 2013
HMC-1	mast cell leukaemia	m	monoculture, co-culture	inflammation	Butterfield *et al.*, 1988
HL-60	acute promyelocytic leukaemia	f	monoculture	cytotoxicity	Bregoli *et al.*, 2009
Mono Mac 6	acute monocytic leukaemia	m	monoculture, co-culture	inflammation	Brown *et al.*, 2000
Airway macrophages	primary peripheral blood cells	m/f	co-culture	particle transport, inflammation	Rothen-Rutishauser *et al.*, 2005
Dendritic cells	primary peripheral blood cells	m/f	co-culture	particle transport, inflammation	Rothen-Rutishauser *et al.*, 2005
Other cells					
fibroblasts	primary fibroblasts	m/f	co-culture	particle toxicity	Hana *et al.*, 2020

References do not show the first citation but show rather key publications

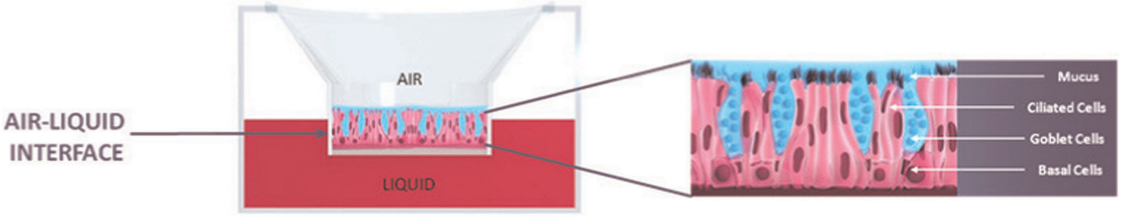

Fig. 9 Representation of the three cell types in MucilAir™: basal, ciliated and goblet cells. Courtesy of Epithelix.

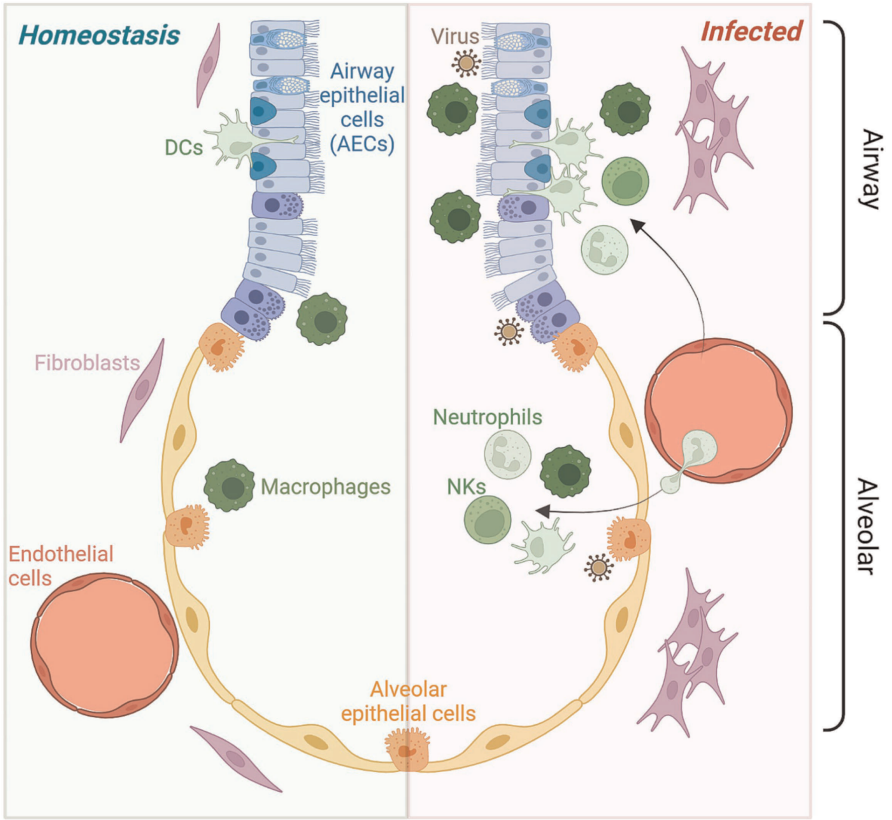

Fig. 10 Summary of the cell types and functions of ideal iPSC-derived *in vitro* airway and alveolar lung models. The airways and alveoli are distinct compartments with unique epithelial cell types and therefore should be modeled separately. We envisage a modular system whereby the airways or alveoli can be modeled at a homeostatic steady state, as well as an inflamed, infected state for comparison. Our ideal system incorporates stromal and endothelial cells, as well as resident (macrophages and dendritic cell [DC]) and infiltrating (neutrophils and natural kill [NK] cell) immune cells. Coupled with the genetic tractability and scalability afforded by iPSC-derived cell types, the intercellular crosstalk, as well as cell intrinsic processes in physiological contexts can be deconstructed with the complete suite of available molecular and genetic tools. From Turner *et al.* (2024).

9 Stem Cells

Induced pluripotent stem cells (iPSC) have been used to develop lung organoids (Kim *et al.*, 2020) and recently cell-types and what would be needed were reviewed (**Fig. 10**. It must be noted that such models are even less standardized than complex models based on cell lines or primary cells. The actual differentiation processes are complex which probably is the reason for the observed high variation of results. Expensive reagents also increase the costs of such models and the differentiation through the different stages from an iPSC to definitive endoderm, anterior foregut endoderm, early lung progenitors to bronchial or alveolar progenitors are complex and not fully understood, although successfully performed (Hawkins and Kotton, 2015). Meanwhile detailed protocols for produce lung organoids or alveolar epithelial cells from human iPSCs have been published (Miller *et al.*, 2019; Tanabe *et al.*, 2024).

It has been advocated that lung organoid cultures can fill gaps such as in how far they mimic the original tissue, are they reflecting patient diversity and how well the cultured organoids reflect diversity within the patients (**Fig. 11**) (Hughes *et al.*, 2023;

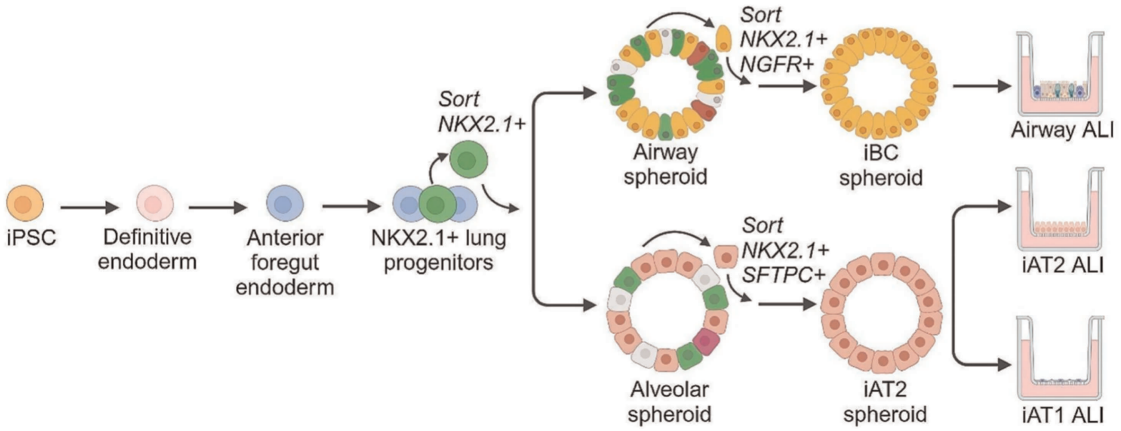

Fig. 11 Schematic of directed differentiation of iPSCs to airway and alveolar epithelium. Differentiation of lung epithelial cells from induced pluripotent stem cell (iPSCs) begins with the induction of definitive endoderm and its subsequent specification to anterior foregut endoderm. Expression of the transcription factor NKX2–1 marks the appearance of bipotent lung progenitor cells, capable of forming both airway epithelial and alveolar epithelial cells. To generate airway epithelial cells, lung progenitors are first differentiated to basal cells that, following enrichment by flow cytometry, can be maintained as spheroids for up to ten passages. Plating these basal cells in air-liquid interface (ALI) cultures with specific differentiation media promotes the formation of a mature pseudostratified epithelium. To generate alveolar epithelial cells, lung progenitors are differentiated to type 2 alveolar epithelial (iAT2) cells using specific growth factors and small molecules. Once formed, iAT2s can also be expanded as 3D spheroids for up to 9 months. When required, iAT2s can be seeded at ALI to induce further maturation. In addition, iAT1 cells can also be generated from iAT2s at ALI using specific media and seeding conditions. From Turner *et al.* (2024).

Turner *et al.*, 2024). A proof-of-concept approach has been recently described to grow patient-specific organoids derived from iPSCs and first results showed expression of lung cell markers (Küstermann *et al.*, 2024). iPSC based models have been used to study immune response to viral infections (Yin *et al.*, 2021). To our best knowledge no such model has been applied for any routine or experimental toxicological evaluation purpose yet. The real open question is of course in how far such organoids that do not offer the possibility of exposure via the airstream can be used for toxicological assessments of airborne toxicants.

10 Organ-on-Chip

As discussed, traditionally pulmonary toxicology was for a long time based on simple submerged cell lines or on *in vivo* experiments using rodents. Significant efforts have been invested in the development of lung-on-a-chip models as a special case of organ-on-a-chip (OoC) technology often in combination with ALI exposure conditions, which also allow to mimic the breathing motion, and the forces exerted on cells under *in vitro* conditions (Park *et al.*, 2024). In OoC models for the respiratory tract usually a membrane, often made of a flexible material, separates the upper compartment where cells are cultured at the ALI mimicking the airflow in the lungs. Below the (elastic) membrane of such an OoC system cell culture medium is circulating mimicking the blood flow.

Adding airflow to the system when culturing epithelial cells under ALI conditions using a Chip-S1® device (Emulate Bio, US) induced cell differentiation and reduced the baseline secretion of interleukin-8 and other inflammatory markers (Nawroth *et al.*, 2023). Epithelial airway cells showed changes in the mucociliary differentiation when bi-directional airflow mimicking conditions during inhalation and exhalation was applied compared to unidirectional airflow (Park *et al.*, 2023). A model in which cyclic stretch has been applied showed an increased resemblance of the molecular processes at the human lung compared to the static condition (Stucki *et al*, 2015). Later additional improvements to the system were made and measurement of biological markers showed high relevance of the cells growing on the stretched membranes (Zamprogno *et al.*, 2021). A fully developed model is already commercially available (Alveolix, Bern, CH). Mechanical stretching resulted in increased cell invasion in A549 cells and increased expression of tumour necrosis factor-alpha (TNF-α) protein (Chen *et al.*, 2023). This is an indication that models applying stretch forces may be better models for tumour invasion. Exposure of primary endothelial cells (HUVEC), widely used in pulmonary co-culture models cultured under physiological stretching to silica nanoparticles did not changing the toxic impact but is reducing the uptake of the nanoparticles, which may have implications for medically active nanoparticles in a therapeutic setting (Freese *et al.*, 2014).

It must be noted that not only OoCs for lung tissue have been developed but recently also aa nasal airway-o-chip has been developed. Again, cells under flow conditions were behaving differently and showed decreased morphological changes and mucus secretion but also decreased inflammation (Walls *et al.*, 2024).

Such OoC models with relevance for the lung have increasingly become commercially available. A good overview on providers was recently published (Park *et al.*, 2024). Reader of this chapter should be able to localize more or younger companies in the future using their favourite browser or on relevant databases (see "Relevant Websites" section).

There are many existing alternatives described above to develop complex cellular *in vitro* models. Many consider the use of microfluidics coupled with co-cultivation of different cell types representing the state-of-the-art for *in vitro* models. More than a decade ago the first "lung on-a-chip" platform, composed of human alveolar epithelial cells and human pulmonary endothelial cells cultivated on the two different sides of a flexible porous membrane was reported (Huh *et al.*, 2010). The system consisted of a flexible membrane in-between compartmentalized channels allowing on the upper side the flow of air and the culture of cells at the ALI and in the lower channel the flow of cell culture medium. This model mimicked also the breathing motion by applying positive and negative pressures in two side channels like what occurs *in vivo* during breathing. Exposure to NPs respectively bacteria resulted in clear differences between static and dynamic conditions.

However, some serious limitations of OoC system representing the pulmonary system must be mentioned. The costs of such OoC systems are still prohibitively high and standardisation is mostly lacking. Controlled exposure is very difficult in most systems and deposition of particles is known to depend on aerodynamics so exposure in OoC systems may be far from representative for an *in vivo* situation. Cell culture in very small channels does not allow for easy chemical analysis of the systems to evaluate the deposited dose of a chemical. Accessibility of the cultured cells for further analysis of biological endpoints is also often complex and the procedures to obtain samples may interfere with omics endpoints. Due to the miniaturized architecture cell number is usually too low to allow the evaluation of endpoints using Fluorescence Activated Cell Sorting (FACS) equipment or classical omics technology. The last problem can be overcome by state-of-the-art single cell transcriptomics. However, this is at this moment also a very expensive technology adding to the overall costs.

11 Precision Cut Lung Slices

Although in use since the 1920s, *ex vivo* lung slices have gained increasing traction in research with the advent of precision-cutting instruments (e.g., Krumdieck tissue slicer (Krumdieck *et al.*, 1980), Leica microtomes, etc.) of which many manufacturers now offer varied designs. The tissue slicers can generate uniform and thinly sliced sections of lung tissue, whereby variability in biomass is minimized and allow a more consistent comparison across technical replicates within treatment groups. Regarded as a key improvement in the ability to slice lung tissue and maintain it in culture, Placke and Fisher reported the use of an agarose solution that is filled into the lung, expanding the airways, and allowing it to gel before further processing into slices (Placke and Fisher, 1987) (**Fig. 12**). While human PCLS can be obtained commercially, many laboratories choose to create their own and the general process is similar in laboratories disclosing their methods (Liu *et al.*, 2019; Morin *et al.*, 2013; Patel *et al.*, 2023).

Because lungs are typically filled with air *in vivo*, PCLS are quite distinct from other organ slices in terms of how they are created. Lungs are inflated with a warm, physiologically compatible, low melting point agarose solution (typically 0.5–3% agarose) (Viana *et al.*, 2022) dispensed into the conducting airways and allowed to gel at cold temperature. Peripheral lung sections are made, tissue cores (typically 8–10 mm in diameter or width) are punched from the sections, and the cores are then inserted into the tissue slicer. Slicing occurs submerged (Krumdieck or Brendel-Vitron-based slicer) in a physiological buffer or in air after mounting onto a vibratome/microtome style slicer. Generated PCLS (~8–10 mm in diameter, and ~150–500 µm in thickness (Viana *et al.*, 2022) are then cultured submerged, embedded in hydrogels, using the dynamic organ roller culture

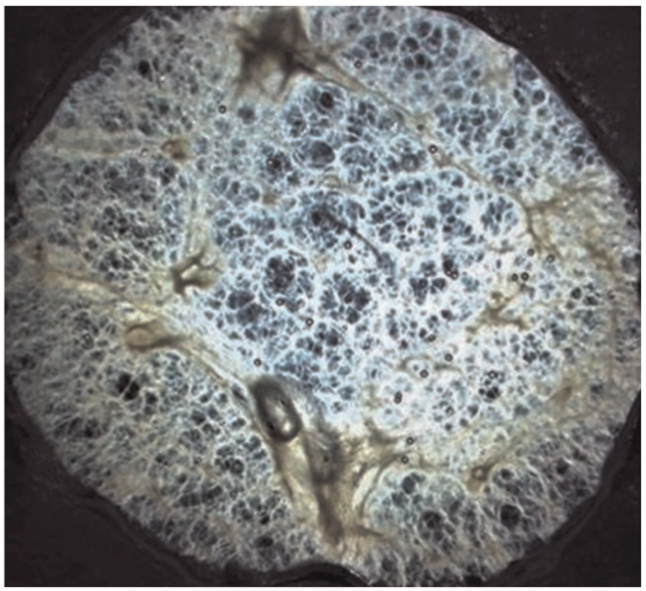

Fig. 12 Human PCLS. Lung slices retain native architecture, (including alveoli and small airways) and all cell types, including immune cells present in the tissue. Courtesy of IIVS

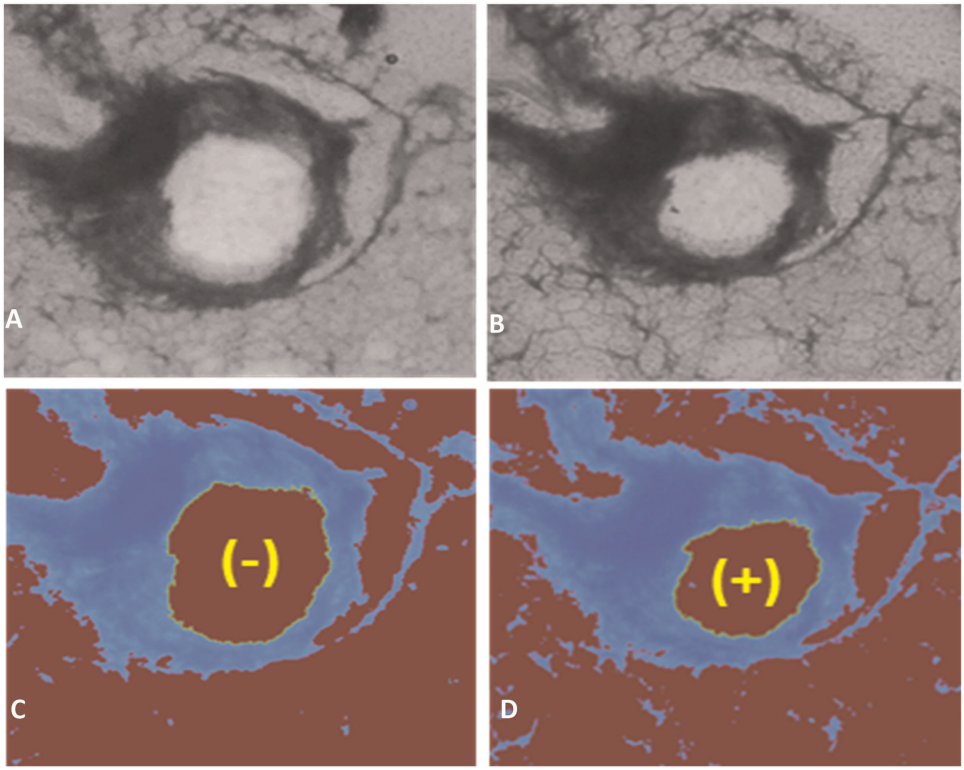

Fig. 13 Human PCLS Airway Contractility. Cross sections of complete small airways with fully exposed lumen are compatible with airway contractility studies. High contrast brightfield images are taken pre- (A) and 15' post- (B) 400 nM methacholine (a bronchoconstrictor) exposure show constriction. Image digitalization, and contrast rendering using Image J allows surface area quantitation pre- (C, yellow (-)) and post (D, yellow (+)) treatment, quantifying a 41.1% decrease in airway lumen area. From Behrsing, H.P. unpubl. results

method (DOC), or at the air-liquid-interface (ALI) using tissue culture inserts in multi-well plates. The specific culture media formulations can vary, but a recent study of human PCLS suggested long-term (4-week) ALI cultures using DMEM:F12-based media was superior to submerged or DOC (Patel *et al.*, 2021). Certainly, the initial lung quality and preparation specifics contribute to the overall performance of PCLS in culture.

As an *ex vivo* tissue, PCLS retain numerous lung attributes including cell types and architecture (i.e., extracellular scaffold). PCLS are comprised of variable airway and alveolar ratios and the physical size of the lung will also limit where PCLS can be derived from (i.e., large lungs relegate the slices to the distal regions containing small airways and alveolar space). Nonetheless, the retention of native architecture and numerous cell types (Stegmayr *et al.*, 2021), including immune cells that can be activated upon tissue challenge is unmatched by other lung models. The retention of agarose in the airway spaces and the cross-section of tissue they represent (i.e., no distinct airway lumen vs epithelial layer orientation) may be viewed as a shortcoming, but it is currently unclear how this adversely impacts tissue responses other than endpoint assays that may not be supported (e.g., trans-epithelial electrical resistance).

However, the cross-section orientation of PCLS often presents a cross section through small airways that boast a compliment of smooth muscle that enables airway contraction studies (**Fig. 13**). Such studies allow the evaluation of asthmatic pharmaceuticals in development, directly in human tissue (normal or diseased). Further, the cross-section orientation and uniform slicing has made this model amenable to tissue stiffness measurements, among many other research applications.

PCLS have been applied for basic research, toxicology, efficacy, disease and infection modelling, etc. Despite conflicting reports about longevity in culture, proper PCLS maintenance conditions can facilitate multi-week cultures and enable long-term assessments of chronic or repeat exposure scenarios (Patel *et al.*, 2021). While most research may focus on short-term, acute events, the ability to study phenomenon like chronic inflammation that have known involvement in many lung diseases creates many opportunities for scientific interrogation. Further, while diseased donor tissue can be obtained to create PCLS, the induction of disease phenotype using normal donor tissue has been reported and offers an alternative to tissue that is inherently scarce (Alsafadi *et al.*, 2017).

A non-exhaustive list of PCLS applications includes physiology (Sanderson, 2011), exposure induced toxicity (Fisher *et al.*, 1994), fibrosis and COPD (Alsafadi *et al.*, 2017; Cedilak *et al.*, 2019; Marimoutou *et al.*, 2024; Westra *et al.*, 2013), inflammation and immunotoxicity (Henjakovic *et al.*, 2008; Lauenstein *et al.*, 2014; Sewald and Braun, 2013; Switalla *et al.*, 2010; Temann *et al.*, 2017), metabolism (Yilmaz *et al.*, 2019), airway contractility (Jude *et al.*, 2016; Jude *et al.*, 2019), transcriptomics (Stegmayr *et al.*, 2021), and vaccine exposure response (Neuhaus *et al.*, 2013).

Perhaps the greatest barrier for routine use of PCLS (especially human), was the availability of suitable lung tissue, and the ability to store it for future use. Suitable human donor lungs may not become available for weeks or months, depending on the acceptance criteria, and then only several hundred PCLS can be produced. Donor comparisons and repeat donor tissue use was not possible, but recent advances in cryopreservation now allow banking of frozen tissue for use as needed (Patel *et al.*, 2023; Watson *et al.*, 2016; Marimoutou *et al.* in press). With the ability to evaluate donor-donor variability, and access banked tissues as needed, the PCLS test system is now suitable for standardization efforts that may lead to downstream validation and incorporation into a research paradigm that regulatory agencies support as a test system for evaluating effects on the lung.

12 Complexity Versus Simplicity

Cell culture models have in recent years become increasingly complex, which was largely driven by the idea to mimic *in vivo* histology as close as possible, assuming that thereby results will better reflect what is observed *in vivo* in humans and thereby finally increasing the relevance of such models (Antoni *et al.*, 2015)

A complex tetra-culture model for the epithelial barrier (Klein *et al.*, 2013, 2017) consisting of epithelial cells, endothelial cells, macrophages and mast cells grown on either side of cell inserts was used as a test case to study the effects of increasing complexity. For this purpose, network perturbation analyses and gene expression data were used to understand the active gene networks in non-exposed models with only one, two, three or all four cell types seeded in the inserts. Gene expression data were analysed using an in-house developed quantitative network-scoring algorithm. In the full model, co-culturing all four cell types the addition of the THP-1 derived macrophages resulted in the largest network perturbations. In addition, increasing similarity of the active gene networks with increasing cell-types present was observed in what is to our best knowledge the most thorough evaluation of the added value of complexity (Marescotti *et al.*, 2019).

In a model consisting of A549 and HULEC-5a cells co-cultured at the ALI expression of type I and type II alveolar epithelial cell markers was reported showing effects not observed in monocultures (Licciardello *et al.*, 2023). Interestingly, TEER values were higher in a co-culture of hAELVi with THP-1 cells than in hAELVi alone in parallel with a decreased permeability for fluorescein showing effects of cell-cell interaction (Kletting *et al.*, 2018). Monocultures, co-cultures and tri-cultures consisting of A549, EA.hy926 and THP-1 cells were exposed to NPs, with these models almost identical with the *in vitro* model evaluated in the study on active gene networks (Marescotti *et al.*, 2019), the monocultures of A549 were more sensitive than multicell models (Azzurra Camassa *et al.*, 2022).

When comparing results between different laboratories using the same model, higher variation was observed for co-culture models (Azzurra Camassa *et al.*, 2022), which underlines the fact of the importance of well-defined standard operation procedures (SOPs) and the application of the principles described further down in the chapter on Good *in vitro* Method Practice (GIVIMP). There was one inter-laboratory exercise performed in which seven laboratories exposed Calu-3 in monoculture or in combination with THP-1 derived macrophages or primary macrophages to an identical set of chemicals using the Vitrocell® Cloud12 system. Results were comparable, and it was concluded that the observed variations are acceptable for endpoints such as cell viability and transepithelial resistance (Braakhuis *et al.*, 2023). The overall conclusion of this publication was that further optimizations steps are needed before such a method can be submitted to OECD.

13 Serum/Animal Product Free Culture Systems

Although cell culture models have increasingly been adopted to replace *in vivo* studies and has become more reliable, it is essential to recognize that most *in vitro* cell culture models still largely depend on animal-derived components.

Over the past decade, there has been growing awareness within the scientific community regarding the use of animal-derived components such as fetal bovine serum (FBS). FBS is considered as a universal cell culture supplement as it allows the growth, proliferation and maintenance of most cell types, but may contain over 1000 components of varying concentrations between batches (van der Valk *et al.*, 2004). At a time when the reproducibility of experimental methods has never been more critical for the validation of *in vitro* models (Hartung *et al.*, 2004), it has become evident that the use of FBS introduces significant reproducibility challenges (van der Valk and Gstraunthaler, 2017). For instance, an inter-laboratory study was recently performed using the A549 cell line in which the same detailed protocol was applied for submerged and ALI conditions by two laboratories (Barosova *et al.*, 2021). The experimental variability in the cell responses reported, was linked to the use of different batches of FBS, thus limiting the inter-laboratory reproducibility. In addition, with strategies like the 3Rs aiming at promoting the replacement of animal testing, the development of *in vitro* methods has soared, leading to a growing demand for FBS. As the FBS market is only being loosely regulated, fraudulent practices have been reported, such as the addition of adult bovine serum albumin (BSA) or the use of FBS sourced from different countries of origin, which exacerbate variability issues in research outcomes (Gstraunthaler *et al.*, 2014). Finally, it is important to clarify that FBS is sourced from unborn calves at slaughterhouses (2–3 animals for 1 liter of FBS) and the process of harvesting it may cause significant distress to mother cow and kills the fetuses, raising serious ethical concerns regarding animal welfare (Jochems *et al.*, 2002). These challenges emphasize the need for developing and adopting more ethical and reliable *in vitro* alternatives that enhance both the scientific validity and ethical standing of *in vitro* research.

Regarding lung *in vitro* models, progress has already made with the replacement of FBS in cell culture media. The A549 cell line was successfully transitioned to two FBS-free commercially available media (Chary *et al.*, 2022). The evaluation of the morphology, performance and functionality of the cell line following the transition to FBS-free conditions was carefully evaluated. While one media formulation promoted the proliferation and preservation of the phenotype of the cells similar to FBS conditions, the other media lead to decreased growth rate, heterogeneous cell sizes, increased sensitivity to toxicants, and differential gene expression suggesting the differentiation towards alveolar type I (ATI) and ATII epithelial cell phenotypes, thereby resembling closer the *in vivo* conditions (Chary *et al.*, 2022). Similarly, BEAS-2B cells were grown in FBS- and bovine pituitary extract (BPE)- free conditions, in a fully animal-free environment using a microfluidic-based device allowing the dynamic exposure of the cells to NPs (Gupta *et al.*, 2021). Culture conditions proved to have a significant impact on the cellular uptake of nanoplastics. Therefore, traditional cell culture methods (including the use of FBS) may not accurately reflect the uptake of low-density particles such as nanoplastics, potentially leading to an underestimation of their effects on the cells (Gupta *et al.*, 2021). This evidence suggests that growing cells in an environment containing calf proteins may show altered response compared to more realistic animal-free conditions. This highlights the importance of reconsidering the use of animal-derived components as they may hide important biological responses. Efforts are ongoing in the culture of other lung cells. For instance, the Calu-3 cell line was successfully cultured by decreasing to 2.5% the percentage of FBS in basal medium, instead of the 10–15% usually recommended (Kreft *et al.*, 2015), suggesting the potential for further reduction in animal-derived components.

The THP-1 cell line was used in coculture with lung epithelial cells to mimic the lung tissue, serving as a model of dendritic-like cells (Chary *et al.*, 2019) or macrophages-like cells (Braakhuis *et al.*, 2023; Klein *et al.*, 2013). Although the differentiation into macrophages-like cells has not been yet verified following transition, this cell line was successfully adapted and well characterized under FBS-free conditions as part of efforts to transition the OECD validated h-CLAT (human cell line activation test) for skin sensitization testing (Edwards *et al.*, 2018; Marigliani *et al.*, 2019; OECD, 2017). These adaptations align with regulatory requirements for fully human *in vitro* test systems.

FBS is just one of many animal-derived components still widely used in cell culture. Similar concerns apply to porcine trypsin, animal-generated antibodies, BSA, various extracellular matrix components such as collagen from rat tails or matrigel derived from mice with large tumors (Engelbert Strauss sarcoma) and others. These may be replaced by human-derived (e.g., human serum albumin), recombinant, or synthetic substances (e.g., TrypLE). Antibodies in particular are a crucial tool in laboratories across many scientific disciplines. Traditionally antibodies are generated by animal immunization. Similar to FBS, animal-generated antibodies present both ethical and scientific concerns which can be addressed by using animal-free recombinant antibodies (Groff *et al.*, 2024; Viegas Barroso *et al.*, 2020). These recombinant antibodies, often derived from human antibody libraries, offer improved reproducibility and quality compared to traditional animal-based methods (Gray *et al.*, 2020; Groff *et al.*, 2020).

14 Type of Inserts

Inserts have been around for some while, but it was not before the interest of the community interested in ALI culture of lung and skin cells and these specific conditions focused on using inserts that they were more widely used. Commercially available porous membranes are convenient and available made from different polymers, with different pore sizes and pore densities as well as insert sizes. It must be considered that the membrane material and pore size may influence transport of chemicals through the pores as well as the attachment and movement of cells, in particular immune cells. Careful evaluation of all parameters is paramount for the successful application and choice of the proper type of inserts (Chary *et al.*, 2019).

Inserts used to establish complex cell models described earlier, mostly use a 10–50 μm thick polymer membrane such as polycarbonate or PET into which pore sizes ranging between 0.4 and 8 μm depending on the application are etched using ion beams. Over the years the first generation of synthetic membranes has been replaced with membranes based on polymers allowing excellent attachment of lung cells but also migration of immune cells through the pores (Chary *et al.*, 2019). The transport of diesel particles across a layer of A540 growing on a porous membrane with a pore size of 3 μm was reported to be in the order of ca 1% of the doses for the range of 11–22 $\mu g/cm^2$ (Gunasingam *et al.*, 2024). Such membranes can also be prepared from elastic materials allowing to mimic forces on the cells growing on them (Doryab *et al.*, 2019). While most standard membranes are made from stiff and relative thick materials not allowing stretching, an interesting concept of very thin (< μ5 m) bioinspired membranes with increasing wetting properties allowing cycling cell-stretch experiments has recently been developed and applied to *in vitro* models of chronic pulmonary diseases (Doryab *et al.*, 2021). Elastic membranes with pores are also used in LoC systems where cells are mechanically stretched (Stucki *et al.*, 2018). For example, in one of the systems, a 3 μm thin elastic membrane with 3 μm pores that allow for a close co-culture of epithelial and endothelial cells and mechanical stretching is used.

Alternatives to these membranes with etched pores are electro spun membranes based on polymers such as polycaprolactone-gelatine that have a 3D structure in the insert (Licciardello *et al.*, 2023; Krugly *et al.*, 2024).

As a response to the problem of increased global use of petroleum-based plastics, efforts are ongoing to reduce the one-way use of such polymers in the future in cell culture laboratories, and one promising approach is the use of oleogels to replace membrane polymers by bioplastics (Lamanna *et al.*, 2024) or hydrogels from basal lamina components such as collagen and elastin (Zamprogno *et al.*, 2021).

15 Exposure Equipment

Cells may be exposed by submerged exposures (pipetting) or using one of various exposure systems allowing the exposure of cells cultured at the ALI to chemicals, (nano)particles, aerosols or gases. Although submerged exposures are less physiological than exposures to aerosols, they are relatively simple to perform without the need of special equipment. ALI exposure has reached a certain maturity and is currently widely recognized as the better alternative to submerged exposure in respiratory toxicology (Lacroix *et al.*, 2018). However, the need to establish validated methods has been highlighted (Hiemstra *et al.*, 2018).

Several ALI exposure systems have been developed in the last decades. Their technologies have been optimized for specific exposure tasks in relation to organotypic airway tissue models as recently reviewed (Cao *et al.*, 2021).

For the exposure to gases or vapors the most frequently exposure principle is continuous flow. This method provides a continuous low flow of aerosol to the cell cultures with variable exposure duration (e.g., 20 min up to 48 h. A prerequisite is here sufficient availability of test material which can often be a limiting factor, proper dosimetry methods, and experience in aerosol engineering.

Whenever cells are exposed to liquids or particle suspensions, the single droplet sedimentation principle can be used. The advantage of this method is that only small amounts of materials are required, deposition efficiency is high, and performing the exposure is relatively simple.

Dry powders can be exposed under continuous flow if they are available in amounts of several grams. For smallest quantities in the 1–100 milligram range special dry powder sedimentation systems are used – normally for scarce pharmaceuticals compounds or small samples from the environment.

Aerosol sources for continuous flow exposure are gas cylinders, aerosol generators, smoking/vaping machines, atmospheric chambers or directly air from the environment (**Fig. 14**). The main flow rate may range from 0.25–16 l/min., depending on the design of the dilution/distribution system and the characteristics of the aerosol source. In order to obtain a dose-response relation, the main aerosol flow is often diluted with humidified air. To avoid stress for the cell cultures, a slow sample flow of 2 ml/min (24-well sized inserts) or 5 ml/min (6- or 12-well sized inserts) is directed to the cell culture inserts by vacuum with individual flow control (**Fig. 15**). The cell cultures are supplied with media through the membrane of the insert from the basal media compartment.

The exposure devices are heated electronically or by using warm water to physiological relevant temperatures. To ensure readiness for potential contamination or bioaerosol exposure, it is essential that all components are fully autoclavable.

The number of compartments of an exposure device may range from 3 to 96, depending on the throughput requirements. It is important that an exposure is always accompanied by a clean air or vehicle/solvent control exposure. The clean air or vehicle/solvent control serves as a negative control and is essential for the validity of an experiment. The exposure settings should ideally always be optimized in a way, that the control does not harm the cells, and the results should always be compared to a set of

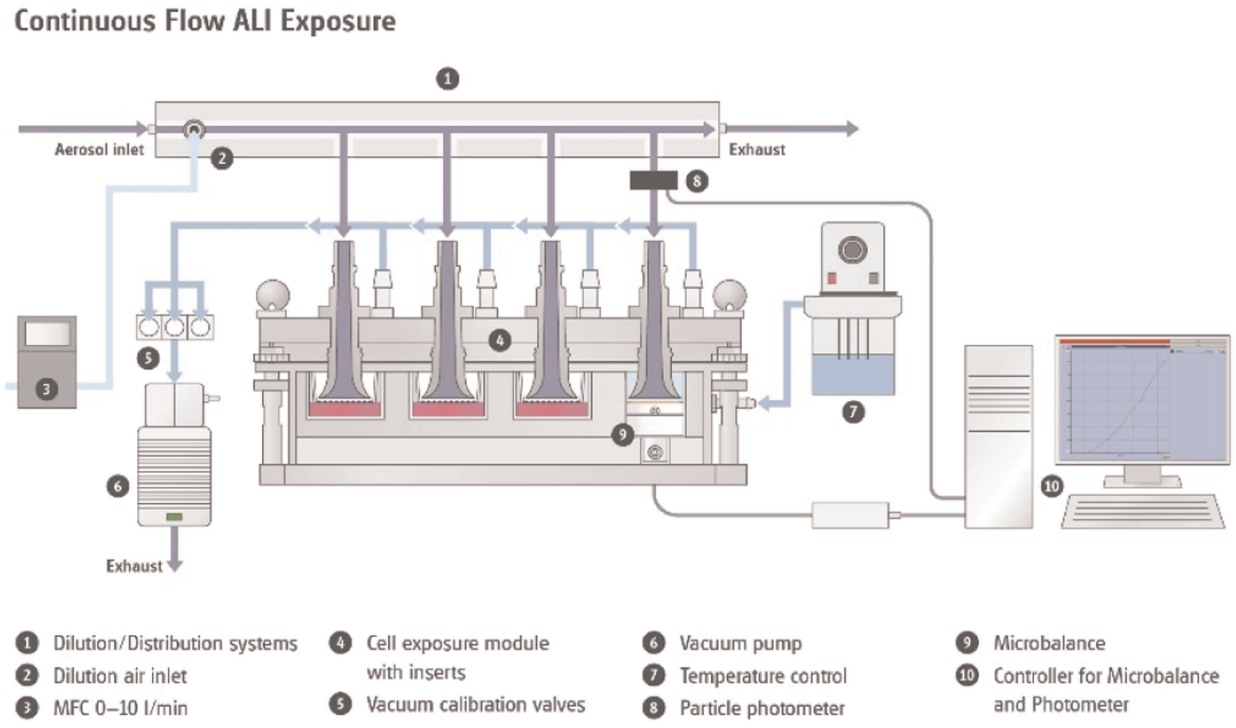

Fig. 14 Flow chart with relevant components of continuous flow exposure system. Courtesy of Vitrocell.

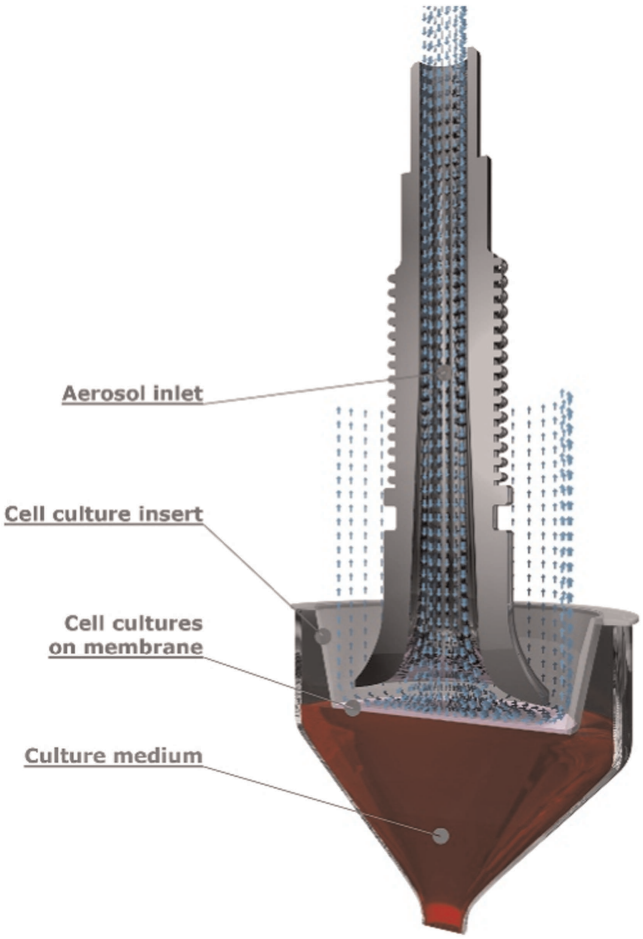

Fig. 15 Detail of flow over cell cultures cultivated on a porous membrane. Courtesy of Vitrocell.

inserts which remained in the incubator at the air-liquid interface to guarantee the exposure itself does not introduce cytotoxicity or other cellular effects. The effects of the exposure to the test substance are evaluated in comparison to the clean air control.

Large systems can accommodate sequential dilutions: a 12-compartment module allows for 3 dilutions with 3 replicates each, alongside 3 replicates for clean air controls. More advanced systems can expose up to 7 dilutions with 7 replicates each, plus 7 clean air replicates. 96-well HTS exposure systems represent the top-end, supporting 11 dilutions with 8 replicates each, in addition to 7 replicates for clean air controls. Such systems have been used to expose cells to poorly soluble nanomaterials and different biological activation levels compared to submerged exposure to suspensions were reported (Loret *et al.*, 2016). Again, validation of such complex systems with many compartments is important and procedures have been described (Keyser *et al.*, 2022).

Special attention should be given to direct a humidified aerosol to cell cultures. For this purpose, special humidification solutions are available (Leibrock *et al.*, 2020). A well-humidified aerosol in the range of 80–90% R.H. enables for realistic exposure conditions and longer exposure duration. As relative humidity depends highly on the surrounding temperature, exposure systems are often housed in chambers which maintain all components at physiological relevant temperatures as this may highly impact robustness (Petersen *et al.*, 2021).

The possibility to use advanced dosimetry tools is a clear advantage of air-liquid interface exposure systems. In addition, such systems offer the possibility to analyze the test aerosol at the cellular level, which is essential to draw conclusions about the deposited dose, particle size and shape, and chemical composition.

To characterize aerosols, gas or particle analyzers can be installed inline. A good example are inline photometers, which can be utilized for real-time measurement of aerosol concentration and such systems have been shown to be useful for extensive physico-chemical characterization of ship engine emissions (Oeder *et al.*, 2015). For the measurement of the deposited mass, Quartz Crystal Microbalances (QCMs) are installed in one of the exposure positions, replacing the cell culture insert, recording the deposited mass in ng/cm^2. The use of a QCMs as online dose measurement during an exposure at the air-liquid interface with nanoparticles were already described in 2009 (Mülhopt *et al.*, 2009). As an alternative, such a compartment can also be equipped with transmission electron microscopy (TEM)-grid holders to study particle size and morphology via transmission electron microscopy (TEM) (Mülhopt *et al.*, 2020).

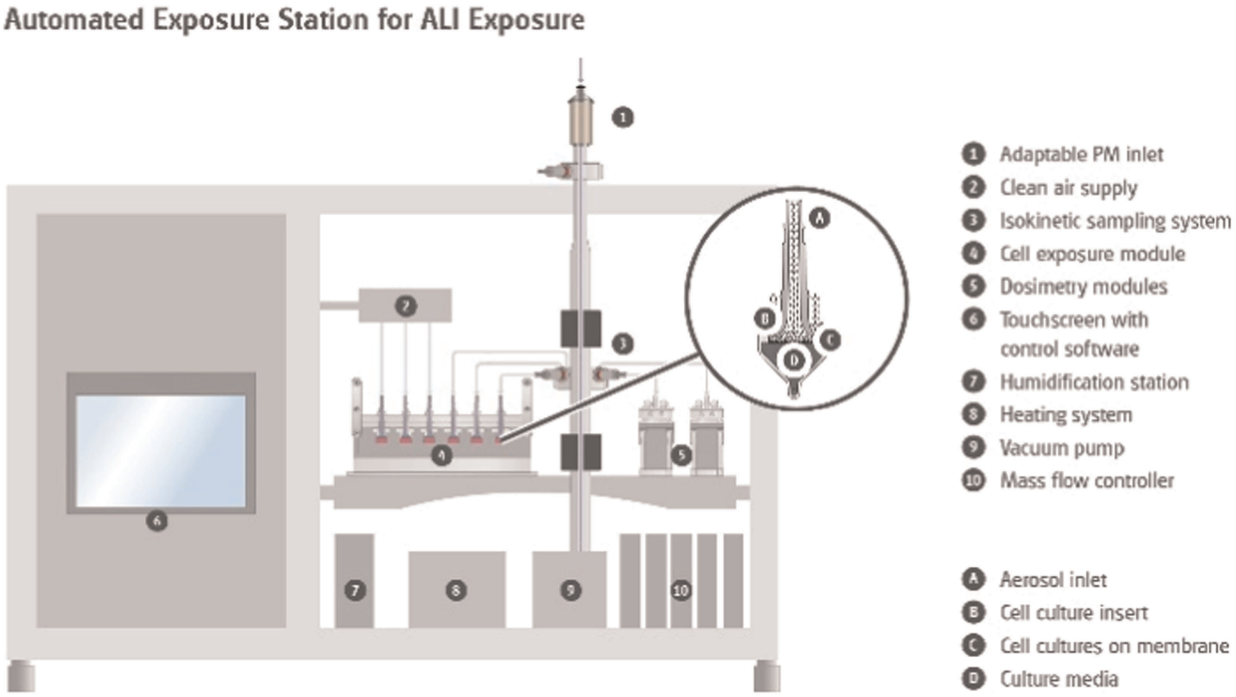

Fig. 16 Flow Chart of an automated exposure system. Courtesy of Vitrocell.

Stainless steel inserts may also be used to trap constituents in liquids for downstream analysis of chemical composition or for assessment of deposition efficiencies by fluorescence (Keyser *et al.*, 2023).

Longer exposure durations, for up to 48 h, may be required for e.g., environmental test concentrations where doses are relatively low or fluctuate during the test period. For this application, exposure devices are required that ensure a stable and controlled environment for aerosol conditioning and cell culture exposure. This is achieved by using automated systems that provide a reproducible and controlled exposure environment while independently regulating all relevant process parameters.

Another benefit of automated systems is a more user-friendly operation which is particularly valuable when a larger group of changing users shares a system. Automated systems provide normally a software supported operation where the user is guided through the experiment (**Fig. 16**).

Process relevant parameters such as chamber temperature, aerosol humidity and flow rates are controlled in the Karlsruhe Exposure System and automated cell exposure for up to 8 h has been reported (Mülhopt *et al.*, 2009). This model has been used to investigate system toxicology of complex wood smoke aerosol using an automated exposure system for cell exposure and determination of cell delivered dose (Dilger *et al.*, 2023). The effects of freshly generated carbon nanoparticles generated by spark ablation (Stermann *et al.*, 2022) and those of differentially treated carbon fibers were studied successfully in the same system (Friesen *et al.*, 2023). This is remarkable as both aerosols are challenging in the aerosol production and transportation from aerosol source to cell culture surface. Effects of atmospheric ageing on the toxicity of soot particles was reported when cell exposure was accompanied by extensive chemical and physical measurements in the system and aerosol supply (Offer *et al.*, 2022).

Another approach to cell exposure at the ALI is the exposure towards single droplets generated from a liquid. In contrast to a continuous aerosol stream, a defined quantity of test liquid is aerosolized into fine droplets using a nebulizer and introduced into a closed chamber. Here, the droplets deposit on the cells through gravitational settling (Lenz *et al.*, 2009) (**Fig. 17**). This approach is ideal for limited or costly materials, such as novel drug candidates, liquid chemicals (Schmid *et al.*, 2017), stable (nano) particle suspensions (Bredeck *et al.*, 2023; Hufnagel *et al.*, 2020), and virus (Bovard *et al.*, 2022). High output rates of the nebulizer are required to induce a formation of vortices to uniformly distribute the generated single droplets within the aerosol chamber (Lenz *et al.*, 2014). Subsequently, these droplets sediment onto the cells within approximately 5–10 min according to their aerodynamic radius, forming a thin layer on top of the cell cultures (Bannuscher *et al.*, 2022). For the duration of exposure, the cells are embedded in heated compartments filled with cell culture media. Various geometries can be implemented to accommodate different cell culture inserts, ranging from 6-well to 12- and 24-well formats, as well as cell HTS culture plates in 24- and 96-well formats (**Fig. 18**).VITROCELL CLOUD." type="start" label="TagFloatf0095">

Novel adaptations of the technology allow for semi-automated systems, where sequential nebulization of the test substance combined with a sliding mechanism enables an easy determination of dose-response relationships (Kohl *et al.*, 2023).

Similar to previously described exposure equipment dosimetry inserts can be integrated, as well as TEM grids or a quartz microbalance (Ding *et al.*, 2020).

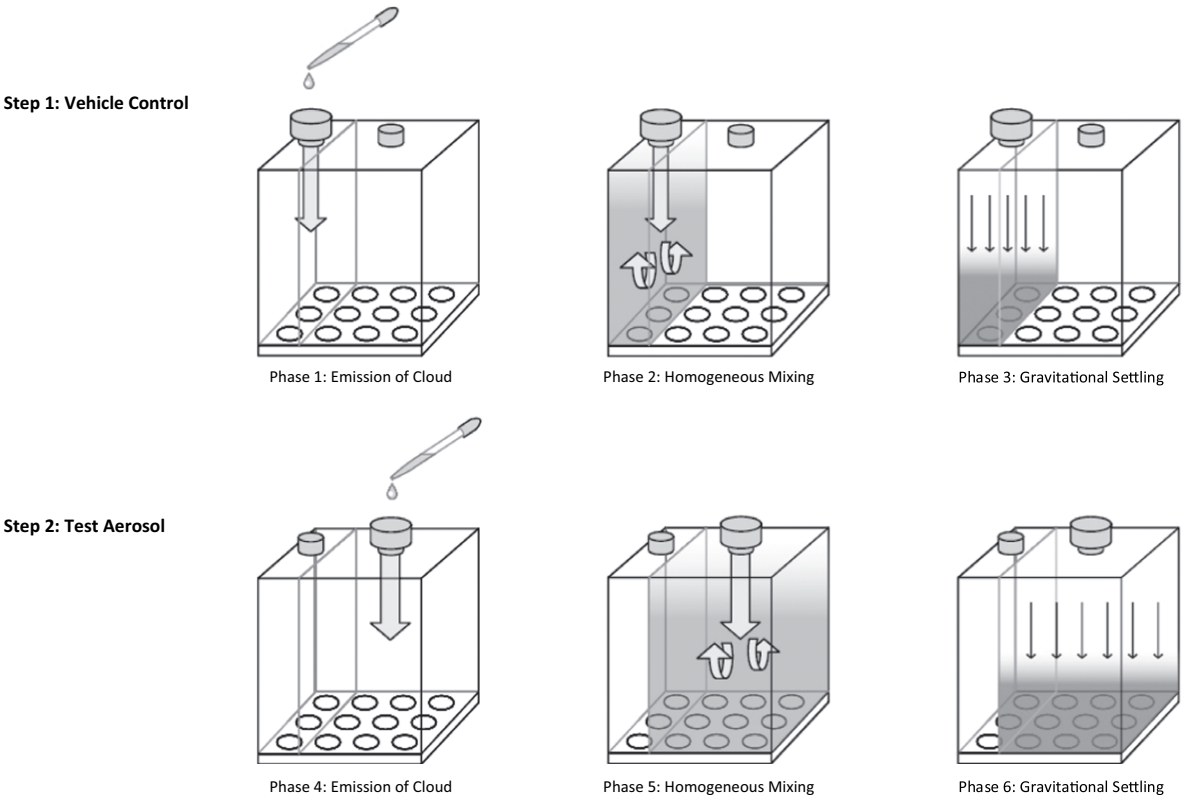

Fig. 17 Schematic workflow of single droplet exposure on ALI cell cultures: Step 1 (top row) illustrates exposure to the vehicle control. Following the emission of the droplet aerosol (1), vortex formation occurs, facilitating the homogenous mixing of the aerosol (2). In the third phase (3), gravitational settling takes place, and the substance deposits on the cells. Simultaneously, during step 2, the test substance is dispended in the same manner. Courtesy of Vitrocell

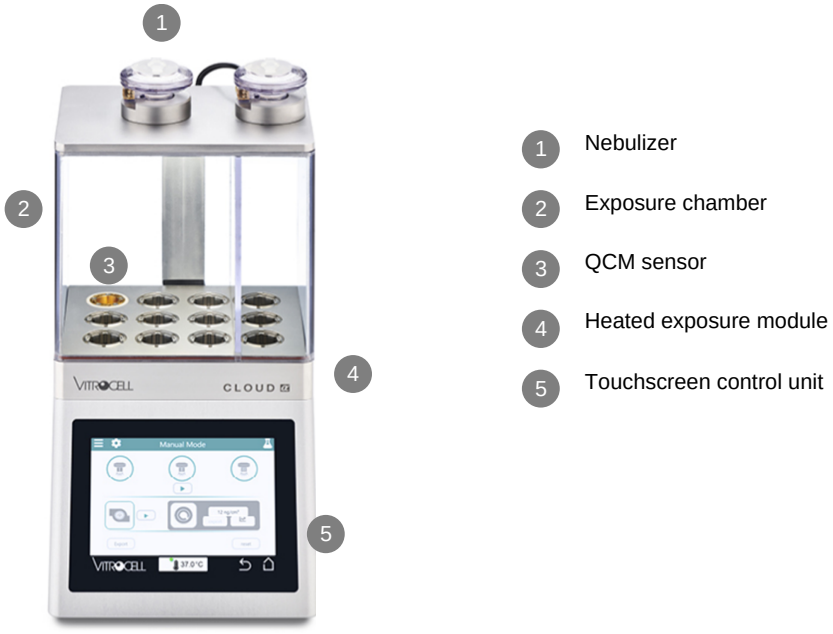

Fig. 18 VITROCELL Cloud System for single droplet sedimentation exposure. courtesy of Vitrocell.

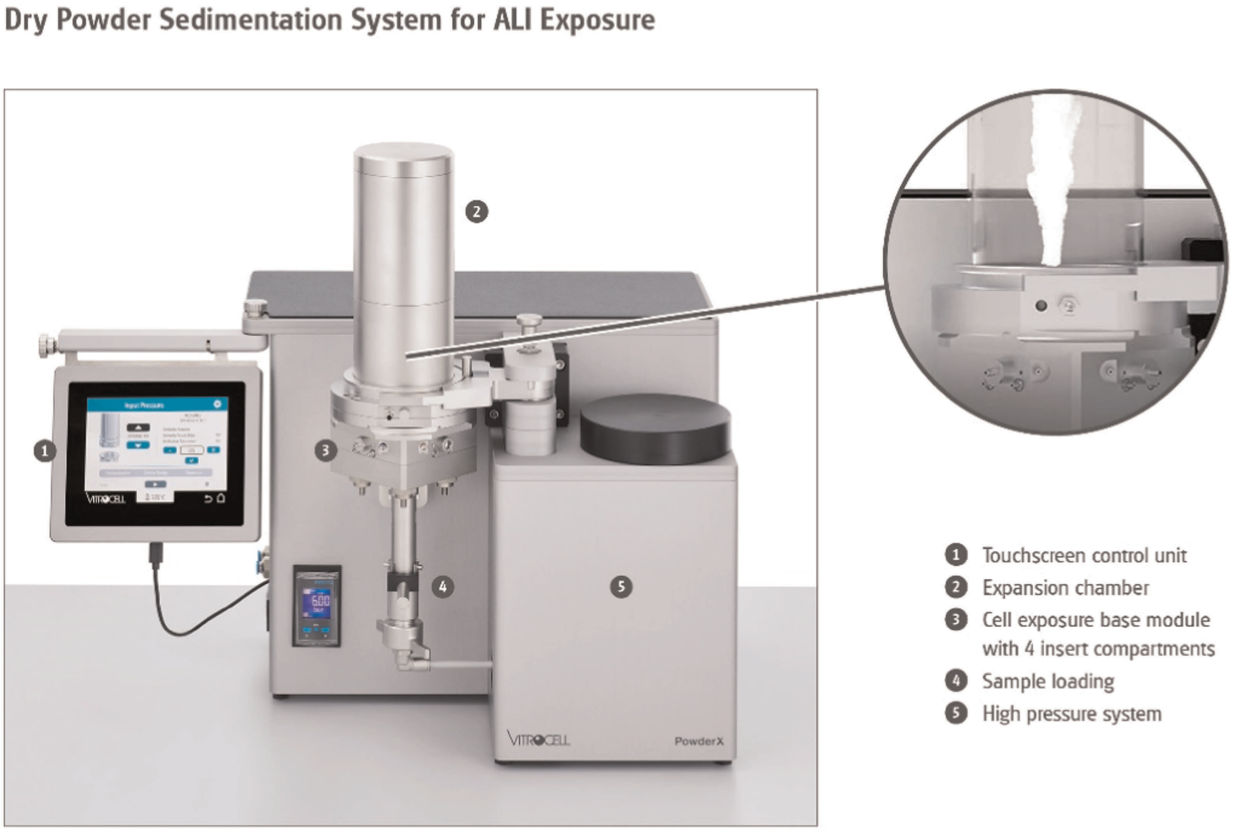

Fig. 19 Dry powder system and components. courtesy of Vitrocell.

Whenever test materials in powder form cannot be suspended in liquids or exposure to powders are more relevant, they need to be aerosolized in their original dry state. There are several solutions to aerosolize dry powders such as e.g., rotating brush generators using a constant airflow. These are well-proven methods in combination with continuous flow exposure. The disadvantage is that they consume a significant amount of test material (several gram). Furthermore, the setup in conjunction with continuous flow exposure is more complex and thus requires highly experienced and trained users. Whenever substances are only available in limited quantities (mg range) as it is the case for expensive pharmaceutical compounds in development stage or for samples collected from the environment, a direct deposition of the test material onto the cell cultures is required. The challenge is here a uniform deposition among the exposed cell culture compartments as well as a homogeneous spread on the surface of the cell culture insert.

A solution to this research task is a direct sedimentation of the aerosolized substance – similar the single droplet sedimentation exposure, but as a dry aerosol (**Fig. 19**). There are also options for sampling from the basolateral media compartment to carry out pharmacokinetic studies.

In recent years a multiplex inhalation platform has been developed that allows the exposure of cells growing in a 96-well format at the ALI under application of sheer forces mimicking very closely the *in vivo* situation including breathing and commercially available (Alveolix, Bern, CH) (Sengupta *et al.*, 2023). This model has recently been applied to study effects of anti-inflammatory drugs (Richter *et al.*, 2024).

Another cell culture exposure system uses horizontal air stream rather than vertical air stream to expose the cells (Zavala *et al.*, 2018). This system is commercialized as CelTox Sampler (MedTec BioLab, Durham, US) and has for example been applied to e-cigarette aerosol (Beard *et al.*, 2024) or gases (Guenétte *et al.*, 2022).

The P.R.I.T.® ExpoCube® was specifically developed to allow exposure of *in vitro* models for air/liquid interface exposure (Ritter *et al.*, 2018) and is commercially available (Scireq, Montreal, CDN). That instrument was used to evaluate toxicity of consumer care products (Ritter *et al.*, 2018) or indoor air (Ritter *et al.*, 2023).

Another system using an exposure chamber containing cells grown on inserts is the CULTEX (Aufderheide and Mohr, 1999). Like many exposure systems the first application of CULTEX was cigarette smoke (Aufderheide *et al.*, 2001). Systems are commercially available (Cultex, Hannover, DE).

Another exposure system that will enable the exposure of inserts at the ALI to airborne agents is the NAVETTA, which has successfully been applied to study the effects if engineered nanoparticles. The system combines horizontal airstream with electrostatic field to allow particle deposition (Frijns *et al.*, 2017).

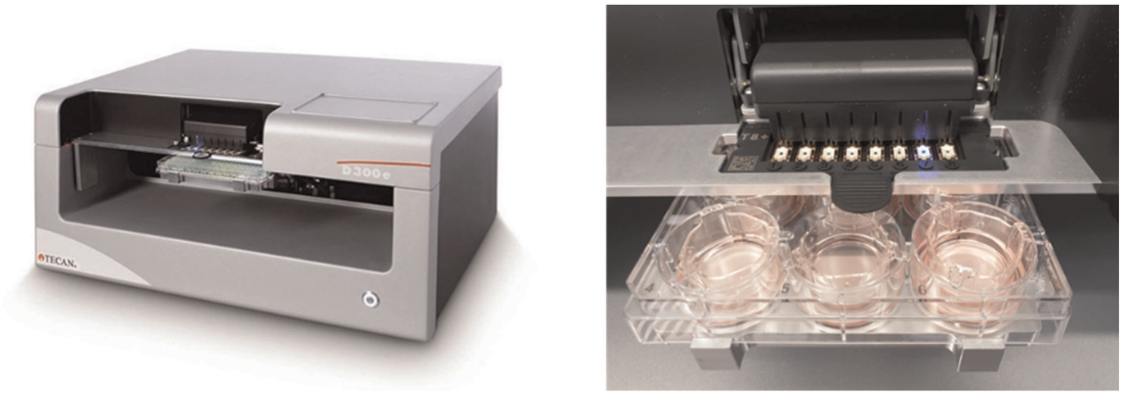

Fig. 20 TECAN D300e digital dispenser (Burla, unpubl.).

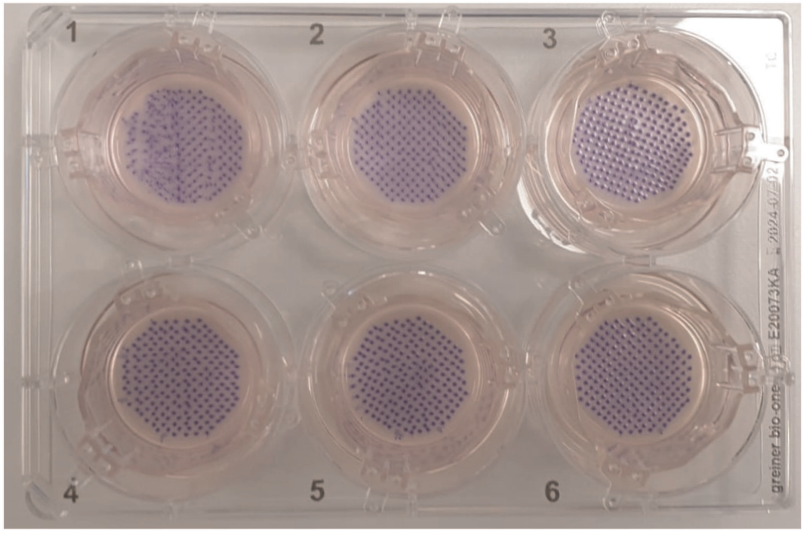

Fig. 21 Dispensing of droplets containing DMP/O on the apical side of the inserts (Burla, see the added reference above).

A system allowing stable and constant exposure of cells to gases has been developed, which enabled ALI exposure of A549 cells to formaldehyde for 3 days (Gostner *et al.*, 2016). This system is currently not commercially available.

In a study evaluating drug application to Calu-3 cells a microsprayer IA-1 C Aerosolizer (PennCentury Inc., Wyndmoor, US; see "Relevant Websites" section) was used to nebulize the tested drugs. In parallel a DP-4 Dry Powder Insufflator (PennCentury Inc., Wyndmoor, US) was used for dry powders (Meindl *et al.*, 2015). It has to be noted that this equipment is not available anymore.

All the above-mentioned exposure tools use gravitational forces sometimes in combination with electrostatic effects for the deposition of the tested materials. Contrary to them the TECAN D300e digital dispenser pipettes defined nanodroplets of the test items solutions on the surface of cells from the apical compartment of the inserts at the ALI (**Fig. 20**) (Burla *et al.*, 2024). For visual inspection of the obtained pattern, a solution of dimethyl phthalate octanol solution with crystal violet (DMP/O) was used as an example to show the delivered droplets on the apical surface of inserts (**Fig. 21**). This systems allows to pipette DMSO and other solvents that cannot be nebulized, while many chemicals cannot be dissolved in PBS or similar hydrophilic solutions.

16 Good In Vitro Method Practices (GIVIMP)

Good *in vitro* Method Practices (GIVIMP) are important for the quality of all aspects of cell culture from development of methods to the application of such methods. A more detailed description of GIVIMP and its added value can be found in chapter x f this book (Ulrey 2025 Standards of Good Practice for the Conduct of *In Vitro* Toxicity Studies, number?).

As discussed in this chapter, a variety of test system types are being developed and routinely used for the *in vitro* assessment of effects of chemicals and materials following inhalation on the lungs. Where primary cells or ex vivo human tissue are used for

assessment, it is important to assure that the cells or tissue has been obtained in an ethical manner (Stacey *et al.*, 2016). Documentation of donor consent should be retained for the protection of the methods and data derived using these test systems. Donor information must be managed appropriately according to applicable national legislation to protect the donor's personal data (OECD, 2018). Researchers should be aware that there may be additional legislation that must be followed when using human-derived calls and tissues, such as the Human Tissue Act of 2004 that is applicable in the United Kingdom (Human Tissue Act, 2004). It also may be important to know the procedures used for procurement (OECD, 2018). Certain harvesting and processing techniques, preparation steps, and transportation conditions may affect the performance of the test system. This information can be obtained by the procurement agency or in cases where the test system is purchased in a prepared state, from the test system supplier. Commercial suppliers of test systems can also provide quality control documents on their systems that includes screening for human pathogens and especially virus and screening for contaminants such as mycoplasma and lot release criteria documenting the acceptability of the system for use. It is helpful if these suppliers follow a quality system themselves, such as GIVIMP, to help ensure the consistency of their systems over time (OECD, 2018). Test system suppliers should also provide laboratories with information on proper safety precautions to take when using their system, steps for proper use of the test system, and instructions for safe disposal. If there are Intellectual Property Rights to the system or any components of it, they should be understood and the effects on the availability and transferability of the method should be considered, whether the test system is purchased or created in-house for use (OECD, 2018).

The potential for donor-to-donor variability in primary cell and *ex vivo* tissue systems was previously mentioned. The use of control charts is a key quality tool to help understand and characterize each lot of test system, which is especially important where there is an expectation of lot-to-lot variability. For purchased systems, suppliers can provide the normal acceptable functioning ranges of their systems. Even in cases where suppliers provide this information to the laboratory, it is important to run in-house controls concurrently with experiments (OECD, 2018). Shipping conditions can affect the performance of the test system on receipt and concurrent controls assure that the test system as received continues to be acceptable for use.

In order to create useful control charts, scientists should consider the required functionality of the test system. For lung systems some points to consider may be barrier function, viability, ciliary beating, contractility and mucus production. A positive control item should be selected that will have a known positive effect on the functional endpoints of interest (OECD, 2018). Negative controls are substances that are not expected to affect the test system under the conditions of exposure. In test systems where there are multiple cell types in a co-culture, biomarkers and/or functional tests can be used to confirm that the required functionality of the system is in place (OECD, 2018). Thoughtfully selected reference items, like positive and negative controls, should be used as part of these functional tests for characterization of the test system. Positive and negative control values should be compiled in a historical control chart to serve as a basis for determining the acceptability of the test system for use. For more information on the selection of controls and their importance, please reference the chapter on standards of good practice for in vitro systems (Ulrey 2025, this book).

Where the test system is an organ-on-a-chip system, there may be components outside of the biological functioning of the cells that must be properly functioning for the test system to routinely function as expected. Often organ-on-a-chip systems require proper media flow that can be controlled by a pump system. They may also need to be held at specific temperatures. In these cases, the cells, pumps, incubation controls, etc. must be properly functioning in order for the test system to perform as expected. It would be useful to think of the test system as the biological component presented in its final platform. Then standardization and calibration of the equipment controlling the platform in which the cells are presented becomes an essential component of test system qualification and control (OECD, 2018).

In vitro respiratory systems often have sophisticated equipment controlling the mixture of test items with air and exposure of the test items to the test systems. Chapter four of the Good In Vitro Method Practices guidance should be consulted to understand some of the controls to put in place to standardize complex equipment. While a formal validation may not always be necessary or required, there should be standardized and documented procedures for cleaning of the equipment and calibration of the instruments prior to use (OECD, 2018).

While it is often optimal to perform cell culture without the use of antibiotics or antifungals in the media (OECD, 2018), their supplementation in the media may be justified, where *ex vivo* tissue cultures or primary cells are used. In these instances, their use should be documented in the protocol or study plan and potential effects on the method and materials to be tested should be considered. Additionally, there may be highly variable media supplements used in respiratory studies. Use of chemically defined media would standardize this and remove a component of study design that could unintentionally add variability to the method (van der Valk and Gstraunthaler, 2017). Where this is not yet possible, it may be prudent to test each batch of media formulated and verify it is acceptable for use in the method prior to the start of any experimental work (OECD, 2018). Where certain components of the media are known to be critical, strategies such as qualifying and purchasing large lots of this component and purchasing these components from reputable suppliers may be helpful.

17 Regulatory Viewpoint

For safety assessment of chemicals, a set of validated in vitro assays for endpoints such as skin sensitization or phototoxicity have been validated by the OECD and are recommended in Europe by ECHA. However, there is still no single *in vitro* method for inhalation toxicity assessment available within the set of official OECD test guidelines (Metz *et al.*, 2021). Since 2023 work is

ongoing which aims to develop the fundament for a future TG on respiratory sensitization within the OECD framework. In the status report of the EURL ECVAM from 2019 two *in vitro* assays with relevance for inhalation are mentioned: 1) EpiAirway™ system to identify acute inhalation toxic chemicals, and 2) ALIsens®, to identify respiratory sensitizers (EURL ECVAM, 2019). EpiAirway started in house pre-validation and aims at regulatory acceptance (Jackson *et al.*, 2018). The Calu-3 epithelium cytotoxicity assay also aims at regulatory acceptance (Jeong *et al.*, 2021).

Generally, the quality of *in vitro* assays and their regulatory readiness needs assessment in respect of their (1) compatibility with regulatory frameworks (i.e., they enable the assessment of an endpoint of regulatory relevance with equal or higher sensitivity and efficiency) and (2) usefulness and usability by the industries complying with regulations and regulatory requirements. This is not trivial and will require that such assays are Transparent, Reliable, Accessible, Applicable and Complete (TRAAC framework) (Shandilya *et al.*, 2023). The regulatory readiness of a set of New Approach Methodologies (NAMs) was recently evaluated among which MucilAir and ALIsens using an objective evaluation system (Shandilya *et al.*, 2023) with both assays showing promising scores and high readiness levels (Hristozov *et al.*, 2024).

18 Outlook and Future Trends

In recent years one clearly emerging trend is that *in vitro* models for the pulmonary system have become central in the testing strategies for airborne chemicals, materials and pathogens. In order to make ALI models more realistic and predictive, developers try to include relevant environmental respectively physiological stress factors such as stretch and pressure, changes in oxygen or CO_2 levels, to better mimic natural physiology (Zimmerling *et al.*, 2024). Models have become increasingly complex, and the added value of complexity has been shown (Marescotti *et al.*, 2019). However, complexity is not a value by itself, and models should only be as complex as necessary and as simply as possible. Great effort has been given by the developer community to generate better and more relevant cells, which better mimic the human pulmonary physiology. However, at present no cell line, either immortalized or cancer-derived, can fully reconstruct the complexity of the alveolar barrier. Most of the cell-based models rely on the use of a single epithelial cell type expressing features of both Type I and Type II alveolar epithelial cells. This is partially solved using primary tissue-derived models, where heterogeneous populations are isolated from human biopsies. However, as discussed earlier, *ex vivo* models are expensive and are difficult to use in regulatory framework for routine applications, making further efforts on the development of cell lines necessary to satisfy the market needs.

The community developing *in vitro* models representing the pulmonary system has started to develop models for diseased tissues. This approach should be further intensified in the light of the serious health effects for affected individuals and the social costs of diseases such as chronic obstructive pulmonary disease (COPD) or lung fibrosis.

The scientific community has recently started to discuss the reproducibility crisis of which *in vitro* and *in silico* models are also part. It seems that our community is partly blind to bias such as that we use cell lines of unclear origin, disregard the sex of the donors of the cell lines used and not even backing back from the creation of chimeric *in vitro* models that use both female and male-derived cells (Gutleb and Gutleb, 2023). It is thus necessary that the international community develops guidelines for harmonization of *in vitro* practices, including development, characterization, maintenance and handling of cell lines. Furthermore, it would be beneficial and is highly recommended that toxicology laboratories apply modern monitoring and certification approaches, such as GIVIMP and GLP.

State-of-the-art omics technology will further open new ways to understand effects and interaction on cellular level in complex models. However, at present, NAMs-derived omics data are too complex to be used as such in regulatory framework. Omics results are particularly useful in generating new hypothesis, which, however, is of difficult applicability in a regulatory framework, where it is required for a test method to answer to specific regulatory endpoints.

Toxicology is on the verge to take the next step in its development as a scientific discipline by integrating artificial intelligence into its toolbox (Hartung, 2023). Such models may be able to identify relevant chemicals in a tiered approach using *in silico* and *in vitro* screening to replace animal experiments in the future (Krieger *et al.*, 2021). AI methods can analyse large datasets as they come from omics experiments and thereby create content and meaning where manual human labour would not succeed (Golden *et al.*, 2021).

Making NAMS acceptable for regulatory purposes and being widely accepted by regulators is a challenge, being the biggest barrier the resilience of the toxicological sector to change and to embrace new approaches. Despite this, the overall policy and the scientific community strive to reduce animal experimentation as much as possible with the final aim to replace and avoid the use of all animals and animal products in hazard assessment. This is especially true for the pulmonary system where anatomical and physiological differences strongly limit the transferability of data from animals to humans.

"The way is lung".

19 COI

AC and ACG are coinventors of WO 2018/122219 A1. ACG is the founder and SB an employee of *INVITROLIZE*. AU and HB are employees of IIVS, a not-for-profit contract research organization that offers assays using *in vitro* and *ex vivo* models and GIVIMP services described in this manuscript.

The authors declare that their contribution was provided in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The manuscript was conceived and developed solely by the authors and the views expressed in this article are those of the authors and do not necessarily represent the views or policies of their respective organizations. Mention of trade names or commercial products does not constitute endorsement for use.

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