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Research Article

Investigations of the Biological Effects of Airborne and Inhalable Substances by Cell-Based *In Vitro* Methods: Fundamental Improvements to the ALI Concept

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The state of the art for cell-based *in vitro* investigations of airborne and inhalable material is "air-liquid interface" (ALI) technology. Cell lines, primary cells, complex 3D models, or precision-cut lung slices (PCLS) are used to represent the lung or skin by way of an *in vitro* barrier model. These models have been applied in toxicity or pharmacological testing. However, contrasting with a clear demand for alternative methods, there is still no widely accepted procedure for cell-based *in vitro* testing of inhalable substances. In the light of this, an analysis was undertaken of common drawbacks of current approaches. Hence, the pivotal improvements aimed at were the cellular exposure environment, overall performance and applicability, operability of online investigations during exposure and routine setup. It resulted in an improved device (P.R.I.T. ExpoCube) based on an "all-in-one-plate" concept including all phases of the experiment (cell culture, exposure, and read-out) and all experimental groups (two test gas groups, controls) in one single commercial multiwell plate. Verification of the concept was demonstrated in a first experimental series using reference substances (formaldehyde, ozone, and clean air). The resulting ALI procedure enables the application of inhalable substances and mixtures under highly effective exposure conditions in routine utilization.

1. Introduction

There are many different reasons for using cell-based *in vitro* methods in inhalation toxicology or pharmacology approaches. Some of them belong to the field of mechanistic research (e.g., ADME studies, drug administration via the lung, and "omics" technologies), some of them belong to legislation and regulation (REACH, European chemical legislation), some have economic reasons (time and money, screening approach, and fast results), and some have ethical reasons (animal welfare, "3R" principle). Although toxicological testing of chemicals and pharmacological investigations based on *in vitro* methods have developed very quickly in recent years and often ended up in standardised and accepted procedures [1, 2], this is not the case for the field of research related to airborne material or inhalable substances. For *in vivo* testing, too, inhalational toxicological and pharmacological approaches still represent a challenging field owing to additional tasks in comparison with oral administration, such as generation and application of gaseous or airborne test substances and specific animal and study design-related aspects [3], including animal handling, behaviour, dosimetry, and many more. A similar situation is the case *in vitro*, where factors such as the processing of cellular test systems in proximate contact with inhalable atmospheres also produce additional, demanding experimental tasks compared with the application of water-soluble substances to submerged *in vitro* cell cultures.

Since the studies by Voisin et al. back in the 1970s [4–7], who introduced the culture technique now known as "air-liquid interphase" or "air-liquid interface" (ALI) condition, research has been carried out to set up more advanced models and experimental procedures for relevant investigations of the biological effects of several types of inhalable compound. Recently, Pariselli et al. [8] focused on environmentally relevant volatile organic compounds. The biological action

of nanoparticles has been extensively studied, as reviewed by Grass et al. [9] and Paur et al. [10]. Automobile exhausts [11, 12] have also been studied recently as well as cigarette smoke [13] or pharmacologically active substances [14]. However, the *in vitro* exposure procedures used within these approaches are specific developments for the individual study tasks. They take special demands into account which are given by the test compound or other requirements and are not easily applicable as a routine test system. Hence, up to now, there has been no clearly defined and widely accepted method for conducting an inhalational scientific approach *in vitro* which is applicable as a global routine setup for a larger variety of inhalable substances. Some chief reasons for this situation are analysed in this paper.

In principle, every *in vitro* approach can be structured into the following three main experimental steps:

(1) the choice, preparation, and culture of a *biological test system*;

(2) the *exposure* step, realising an efficient and reproducible contact between the test substance and the biological test system;

(3) the *read-out* step to analyse and quantify the biological effect of the test substance on the biological test systems.

Intensive research has been carried out on steps one and three as global *in vitro* tasks in recent years. Numerous cell-based test systems have been set up for general use and, more specifically, for inhalational research-related topics as well. Single cell-type cultures from cell lines and primary cells from different sources [16], reconstructed complex cell models [17], and even *ex vivo* cell models such as precision-cut lung slices [18] and stem cell derived systems [19] have been established and characterised or are under development. Hence, meanwhile there is a large *in vitro* "toolbox" available. The tools also include biological test systems from different locations, for example, nasal region and upper or lower respiratory tract, for setting up a cell-based test system specifically fitting the relevant target regions of a specific inhalable compound in the lung. Further, methods for the read-out of the biological effect have also been established over a broad range covering all kinds of cellular pathways, including cell toxicity, mechanistic endpoints such as glutathione, chemokine secretion, or changes on the molecular level, and a number of "omics" technologies such as proteomics, metabolomics, and others. Usually, these endpoints can be adapted to most of the available cell-based test systems *in vitro*.

In standard *in vitro* approaches, where, commonly, water-soluble substances are added to submerged cultures, the exposure as the second experimental step is not normally considered to be a critical phase of the experiment, since substance handling and dosimetry take place under conditions that are easy to control. Contrastingly, in inhalation-related cell-based approaches, the generation of the test atmosphere, the establishment of a reproducible, effective, but gentle cellular contact with the exposure atmosphere and complex flow dynamics from aerosols produce challenging experimental tasks.

Historically, there have been many approaches aimed at handling this situation. They have been reviewed regularly [20, 21] and include fundamentally different experimental strategies—with the air-liquid interphase (ALI) technique using mono- or multilayer cultures of adherent biological test systems generally being seen as the most promising exposure strategy in cell-based inhalation approaches. The reasons for this are the relevance and specificity with respect to two main factors. Firstly, ALI test systems represent a relevant biological barrier culture that can model the *in vivo* "first site of contact" organs such as lung and skin very well from the point of view of physiological and biological organisation and function. Secondly, the ALI exposure situation can create a defined, relevant, and effective contact between the biological test system and the inhalable compound from the point of view of fluid dynamics.

The principal ALI technology was recently prevalidated in a round robin study in Germany as a test system for chemical gases [15, 22]. Briefly, the study resulted in a prediction model for acute toxicity with good inter- and intralaboratory reproducibility and a clear discrimination of gases with and without toxic potential. No false-positives were detected. Moreover, a minimum number of control groups (clean air control and nonexposure control) and validation criteria have been defined to validate single exposure experiments.

However, since typical characteristics of current exposure setups lead to limitations in the overall *in vitro* process, the inhalation-related application of ALI technology is still a demanding task for a standard *in vitro* laboratory. Hence, to increase the acceptance and applicability of the method for a larger range of users (beyond just specialists in aerosol sciences), it has to be fundamentally refined to achieve reproducibility, sensitivity, and meaningfulness of results on the one hand and good applicability on the other.

1.1. Design of Cellular Environment and Separation of Exposure and Culture Medium Compartments. The current setups based on ALI technologies can be classified into two fundamental strategies. One type of exposure setup is based on a so-called stagnation flow arrangement where single cultures are exposed individually to an airflow that is directed towards the culture surface [23–25]. This type of setup satisfies the principal requirements for a reproducible and highly efficient exposure of cultures to inhalable substances. On the other hand, these setups always include noncommercial housings for the cultures in order to realise the stagnation flow arrangement. The other type of exposure setup is constructed as a small "incubator-type" arrangement [11, 26, 27] where cells in commercial multiwell plates are positioned in larger housings such as cubic exposure chambers or cell culture incubators. This is a very convenient and reasonable strategy from the point of view of applicability and use as well as mild cellular conditions. On the other hand, it offers only limited capacity for the development of a highly effective exposure situation for cultures from the point of view of flow dynamics and cannot handle single cultures as independent technical replicates. Moreover, it leads to a situation where the exposure aerosol can freely come into contact with the culture medium in these settings. Thus, the exposure compound will also

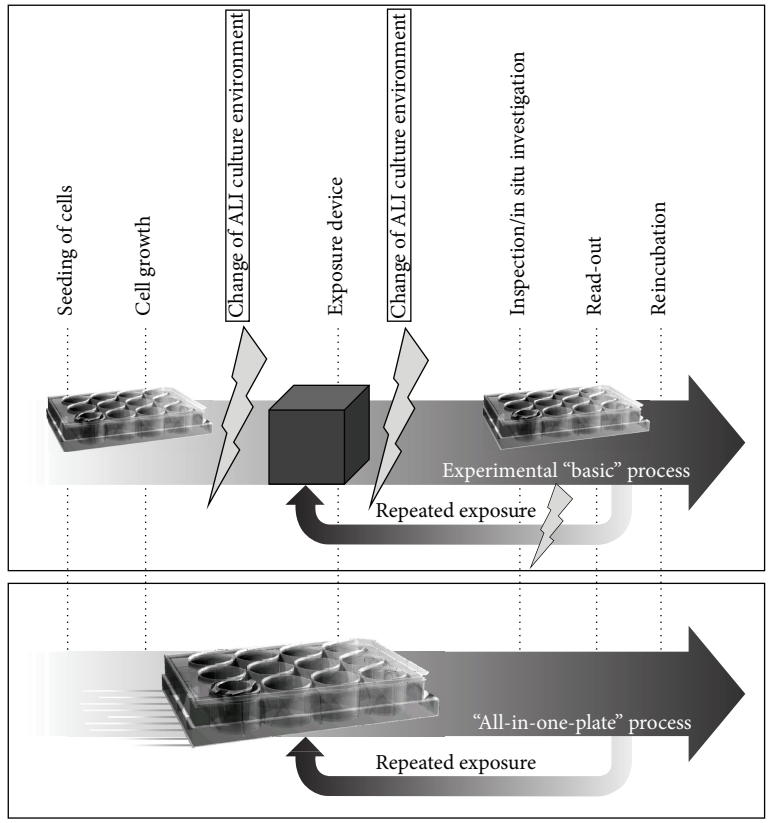


Figure 1: The exposure as an isolated step in the experimental "basic" cell-based process leads to an unfavourable, time-consuming, expensive, and cell-damaging handling of the biological test system in a disrupted workflow in current exposure setups (upper diagram). The "all-in-one-plate" process based on the use of cell culture supports in standard multiwell plates throughout the whole experimental process enables a completely integrated workflow and extended experimental possibilities in the improved ALI procedure.

react with the cells via the culture medium, which is not the intended method of exposure.

1.2. Workflow for In Vitro Testing and Cellular Stress. The use of custom-made housings for the cultures in stagnation flow setups clearly prohibits an integrated workflow for the complete *in vitro* process in inhalation-related cell-based assays (Figure 1). The cultures have to be transferred from standard consumables to the exposure device before exposure and back to standard labware afterwards. At the same time, this change in the cellular environment clearly induces cellular stress and includes the risk of bacterial contamination (since this procedure usually has to take place in nonsterile environments). Also, it is a clear handicap in repeated dose approaches, where cells should be exposed repeatedly after an intermediate nonexposure period.

1.3. Complexity of the Setup, Mobility, Throughput, and Level of Automation. The systems developed so far were in experimental setups that were designed to meet the requirements defined by the test substances of individual studies (such as nanoparticles, smokes, or others). Therefore, the resulting experimental setups vary in complexity and include the individual control of many separate technical elements such as pumps, valves, water baths, and tubing and the location of the setups under hoods or inside culture incubators. In particular, controlling the small exposure flows (commonly <5 mL/min airflow) that have to be applied in stagnation flow setups for single culture exposure is technically demanding, since they are of fundamental importance for the reproducibility of each experimental run. Moreover, typical test materials in inhalation approaches are exposure atmospheres (e.g., motor exhaust, environmental atmospheres, chemical fumes, or others) that cannot be generated inside a cell culture lab in most cases. A certain degree of mobility and user-friendly handling are therefore a critical factor for the use of such setups. Up to now, there has been no technical solution for the elegant handling of all these technical demands. Hence, the current setups can hardly be transferred from laboratory to laboratory for applications to different experimental studies and therefore this complexity and lack of mobility of the setups are a serious limitation in routine applications.

1.4. Observation during Exposure Step. In most cases, the exposure step is a "black box," where no information about the cellular status or cellular changes is accessible. Stagnation flow setups need highly developed and dedicated technical solutions to enable observation of cellular changes during exposure, since they take place in custom-made housings [28, 29]. Setups of the "small incubator type" are also normally not

intended for an inspection of the cultures during exposure. Even occasional cellular inspection by light microscopy during or in between the exposure is usually impossible because of the contamination of the cell support or culture plate with toxic material such as particles or droplets from aerosol exposures.

1.5. Exposure Groups and Controls. The definition and processing of adequate controls are general requirements for the reproducibility and relevance of results. The setups developed so far do not enable a combined and simultaneous exposure of control and test compound groups of cultures.

Finally, one more important topic that has to be focused on in cell-based inhalation testing is the actual particle deposition on the ALI culture surface from test atmospheres containing particles or droplets (particle aerosols or droplet aerosols). However, as this is a complex field of research in itself, it is beyond the scope of this paper.

In the light of this, the present work was carried out to establish fundamental improvements to achieve better reliability, practicability, overall performance, and new perspectives for future developments in cell-based assays related to airborne material. A first set of experiments characterises the resulting "all-in-one-plate" procedure in comparison to the results of the prevalidation study of the basic ALI technology [15, 22].

2. Materials and Methods

2.1. Media, Reagents, and Chemicals. Dulbecco's modified Eagle medium (DMEM) with stable l-Glutamine was supplied by Biochrom (Germany). Medium for cultivation was prepared with 50 μg/mL gentamycin (Gibco, Invitrogen) and 10% foetal calf serum (FCS; Biochrom, Berlin). For exposure experiments, DMEM medium with 15 mM HEPES (Gibco, Invitrogen) and 50 μg/mL gentamycin, but without FCS supplementation, was used. WST-1 solution was purchased from Roche (Mannheim, Germany) and formalin solution from Sigma (Germany).

2.2. Cell Culture. A549-cells (DSMZ, Germany) were used as a human indicator cell line for the alveolar lung region. They were routinely cultured in 75 cm^2 flasks in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal calf serum (FCS). Subconfluent cultures were trypsinised and cell viability was determined using an electronic cell counter (CASY, Schärfe Systems, Germany). Cells were seeded onto polyethylene terephthalate (PET) track-etched membranes (Becton Dickinson, Germany), positioned in a corresponding 12-well multiwell plate, and cultured in an incubator at 95% relative humidity (37°C) and 5% CO_2. Sixteen to eighteen hours before exposure, culture medium was removed from the top of the membranes whereas the basal medium in the related wells was changed to the exposure media.

2.3. Cell Exposure. Shortly before starting the exposure treatment, the multiwell plates housing the cells attached to the membranes were removed from the incubator. Each cell layer was inspected microscopically to check the quality of the preparation. Without transferring the membranes from the corresponding multiwell plate, the remaining media on the top of the cell layers was gently removed by pipetting. Immediately afterwards, the complete multiwell plate containing the membranes was inserted into the well-tempered heating plate of the ExpoCube. The plate lid was removed and the exposure attachment of the ExpoCube was fixed to the culture plate. Subsequently, the exposure to ozone (0.02–17.5 ppm) or alternatively formaldehyde (2–90 ppm) and clean air was started for one hour. Based on the plate layout (Figure 2), cells were either individually exposed to the test gases ozone or formaldehyde on the one line (A) and clean air as a negative reference on the other line (B) simultaneously. Additionally, the same plate included control cultures that were not exposed to lines A or B (nonexposure control).

2.4. Generation of Test Atmospheres and Monitoring of Concentrations. Formaldehyde was generated by conducting clean air over the surface of formalin solutions (35–37% in H_2O) inside a gas washing bottle at a controlled temperature of 22°C. The resulting atmosphere was diluted with clean air until the desired formaldehyde concentration was achieved. The analysis of formaldehyde was carried out by online measurement using an FT-IR analyser (GASMET, Ansyco, Germany), based on the Fourier transform infrared spectroscopy (FTIR) method. Ozone was generated in situ by photolysis of synthetic air using a Penray lamp (Oriel Sarl, Paris) and dilution with clean air. Measurements of ozone concentrations were performed online by an ozone monitor (MLU Modell 400A).

2.5. Measurement of Cellular Viability. The cell proliferation reagent WST-1 is a tetrazolium salt commonly used for spectrophotometric quantification of cellular viability. After exposure, the plate was removed from the ExpoCube, 500 μL WST-1 solution was added apically onto the cells (end concentration 10% v/v in culture medium), and the cells were incubated at 37°C. After one hour, the culture medium from the apical compartment was mixed with the culture medium from the basolateral compartment and transferred in aliquots of 100 μL to 96-well microplates. The absorbance of the formazan solution was determined at 450 nm with a reference wavelength of 630 nm.

2.6. Statistical Calculations. Statistical calculations were carried out using the OriginPro Software package (version 8.1, OriginLab Corporation, USA).

3. Results

3.1. Development of Improved Exposure Device and Exposure Procedure. An improved exposure procedure and exposure device (P.R.I.T. ExpoCube) were developed (Figures 1 and 3). This is the first time that an individual exposure of cultures using a stagnation flow setup and the application of commercially available, usual companion plates has been combined in one device. In a first model of the device,

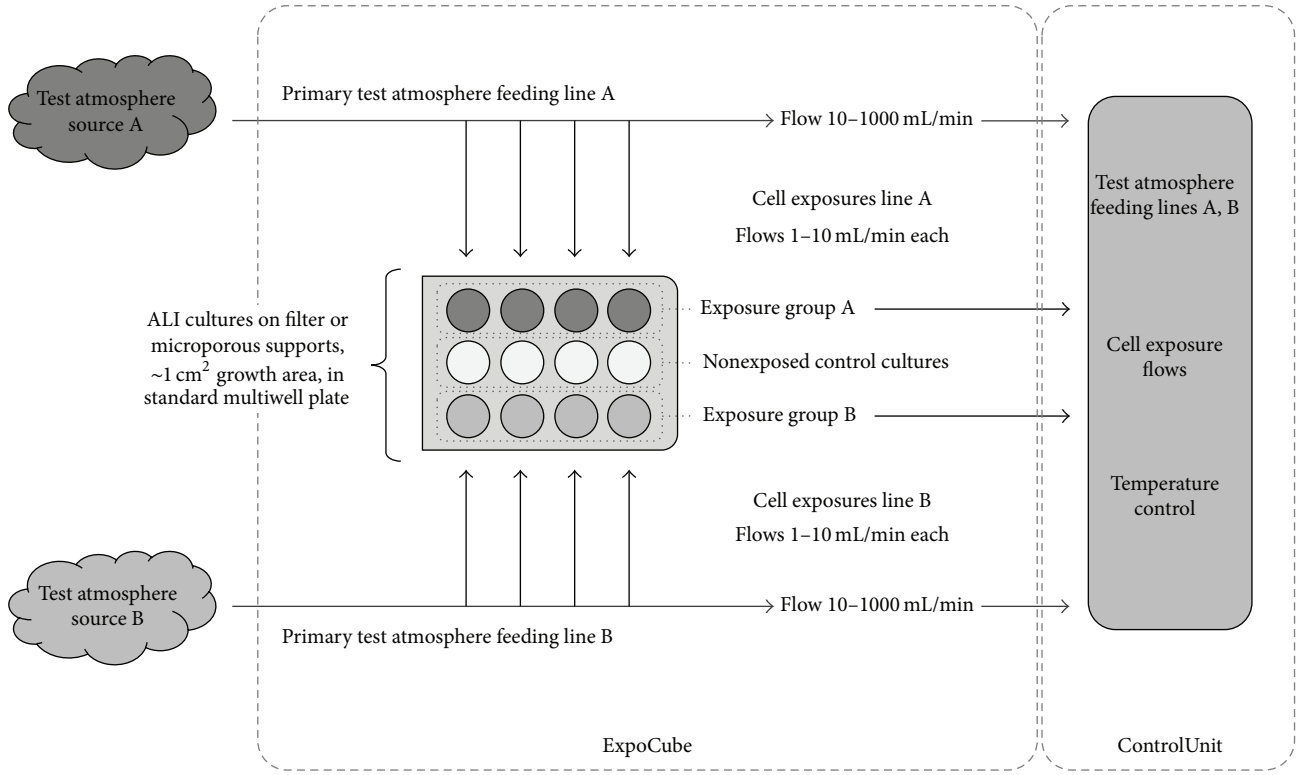


Figure 2: "All-in-one-plate" layout and flow setup of the P.R.I.T. ExpoCube system. The primary test atmosphere feeding flows for exposure lines A and B and the individual exposure flows are controlled by the ControlUnit of the system. The ExpoCube and the ControlUnit provide all the necessary functions and technical components to start a cell-based exposure experiment using airborne substances.

Figure 3: The P.R.I.T. ExpoCube is the result of the development of an improved exposure procedure. Becton Dickinson Falcon culture inserts (left, positioned in multiwell plate) and Corning Transwell permeable supports (right) can be used in 12-well plates.

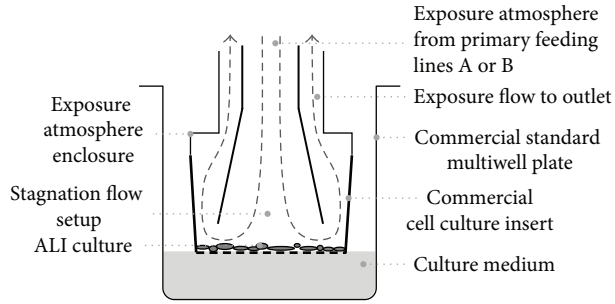


Figure 4: Flow geometry setting inside the ExpoCube including ALI cultures on a microporous membrane in a standard multiwell plate in combination with a stagnation flow setup. The ExpoCube has an exposure atmosphere enclosure that permits any direct contact between the exposure atmosphere and the culture medium.

culture supports of two different types (Becton Dickinson Falcon culture inserts and Corning Transwell permeable supports) with a culture area of about 1 cm^2 were considered. The specific construction of the ExpoCube ensures that the exposure atmosphere and the culture medium are completely separated throughout the experiment (Figure 4).

The ExpoCube was constructed in a modular setup. It includes a temperature-controlled exposure attachment that conducts two different test atmospheres or concentrations from primary feeding lines to the single cultures (Figure 2). Additionally, the culture plate is enclosed and its temperature controlled by a heating plate. The primary exposure atmosphere feed of up to 1000 mL/min continuously transports the test atmosphere to two separate exposure lines by vacuum-driven flows. Hence, any source of test atmospheres, including lab-generated mixtures or aerosols from environmental sources, can easily be applied. From the primary feeding of the test atmosphere, exposure flows are

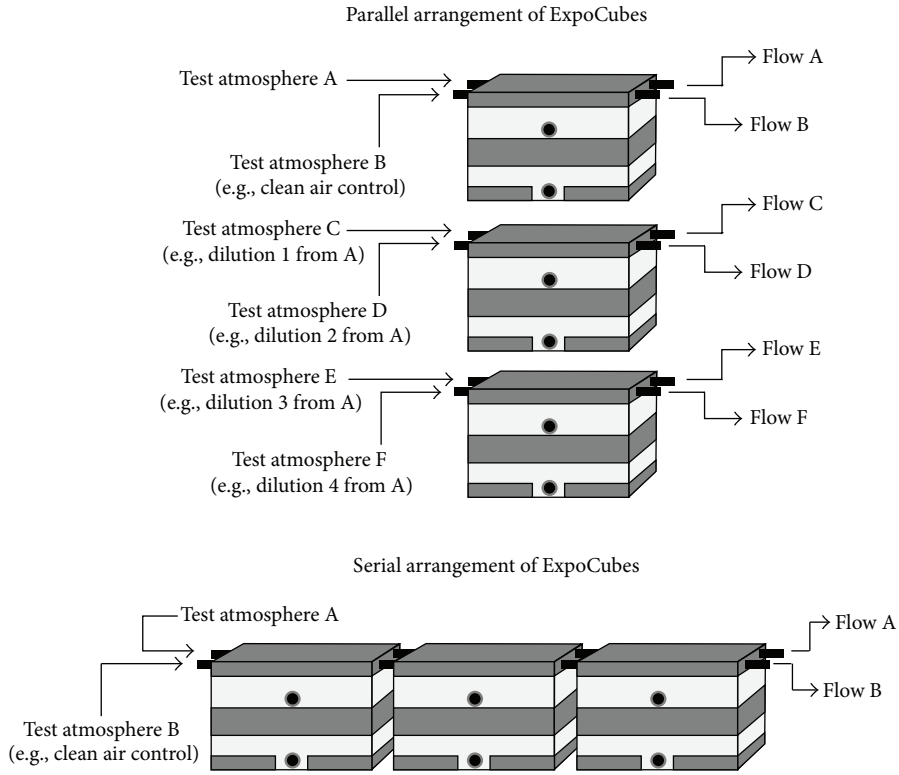


Figure 5: Arranging multiple ExpoCubes to enable a larger number of exposure groups (parallel arrangement, e.g., different test compound concentrations) or a larger number of technical replicates (serial arrangement).

conducted over the surface of the individual ALI cultures using a CFD (computational fluidics design) optimised flow geometry. Depending on exposure conditions for a specific ALI culture system, these flows are usually set in the range of 1–10 mL/min.

The cultures in the consumable multiwell plate are organised by an "all-in-one-plate" approach into three exposure groups (Figure 2). Two groups (exposure groups A and B) can typically be assigned to a test compound group and an exposure control group (e.g., clean air) or different exposure concentrations. An included nonexposure control group is not exposed to airflow. It represents a process validation control for each single exposure experiment. Each of the groups includes four technical replicates. The parts of the device in direct contact with the exposure atmosphere are made from polytetrafluoroethylene or stainless steel. The ExpoCube can be used inline in a flow system. Multiple ExpoCubes can easily be used to manage a higher number of exposure groups (parallel arrangement) or technical replicates (serial arrangement) as shown in Figure 5.

The ExpoCube is driven by a compact 19-inch device (P.R.I.T. ControlUnit), which includes all technical modules to drive the exposure such as an adjustment of the primary test atmosphere feeding lines A and B, the individual exposure flows, and the temperature control by electrical heating (Figure 2). The ExpoCube is small in size (about $20 \times 12 \times 10$ cm). Using the ExpoCube and the ControlUnit only, an immediate laboratory-independent cell-based exposure experiment can be started without any additional technical supports. This setup permits samples of almost every source (ambient air, engine combustion, workplace atmosphere, etc.) to be taken, which allows a sample flow between 10 and 1000 mL/min at atmospheric pressure.

The continuous use of cultures in standard multiwell plates results in a completely integrated "all-in-one-plate" workflow for a cell-based exposure experiment (Figure 1). Cultures are routinely prepared using standard cell-specific conditions and commercial multiwell plates in the incubator. Immediately before exposure, the plates can be inspected by light microscopy and transferred to the ExpoCube without a change in the culture medium or any other manipulation of the cultures or their environment. After exposure, the multiwell plate, including the exposed ALI cultures, can easily be taken off the ExpoCube and handled using the standard protocols for read-out determination or postexposure incubation. It is also possible to take the cultures out and thus intermit the exposure, inspect them microscopically, or manipulate them in any way if necessary and restart the exposure once only or repeatedly with the same culture but without changing the culture medium.

3.2. Exposure to Clean Air, Formaldehyde, and Ozone. A first characterisation of the new exposure procedure involved exposure to clean air, ozone, and formaldehyde. The experimental design was based on the protocol of the prevalidation study for chemical gases using ALI culture systems [15, 22]. It included monolayer ALI cultures from A549 human lung cells and three exposure groups (nonexposure controls, clean

air controls, and the test atmosphere group) and involved an exposure period of one hour and immediate analysis of the cellular viability after the end of the exposure. WST-1 was analysed here as a measure of cellular viability. Formaldehyde and ozone were applied in varying concentrations for the establishment of dose-response relationships, from which EC_{50} values were calculated. The clean air control group served two purposes. On the one hand, a validity control parameter was calculated from the percentage of clean air group viability in comparison to the nonexposed control; a value lower than 70% would indicate an invalid experiment. At the same time, the clean air control served as the reference control for the test atmosphere group for calculating quantitative biological effects as a percentage of control value.

The clean air exposure atmosphere was not humidified (resulting in <20% r.H. @ 22°C) or heated or supplemented by CO_2. The results of 23 clean air exposure experiments are shown in Figure 6. The mean value of clean air viability was 97.05 ± 8.83% of nonexposed controls. Neither a single technical replicate nor the mean of four technical replicates was found to have a value lower than 70% of the nonexposed controls in these experiments, which was defined as the limit of validity during the prevalidation of the method.

Formaldehyde was continuously generated from formaldehyde solutions in water (36.5–38%) and dilution in clean air. The actual concentration was measured online by FT-IR. Compound concentrations between 2 and 90 ppm were generated and applied in six independent exposure experiments. The WST-1 assay as a measure of cellular viability was conducted immediately after 60 minutes of exposure. O.D. readings from formaldehyde exposure groups were calculated as a percentage of control from concurrent clean air exposure groups from the same multiwell plate. Results were plotted against a measure of the dosage calculated from the formaldehyde concentration (ppm), exposure time (60 min), the applied exposure flow (3 mL/min), and cell culture surface area (1 cm^2). Fitting by a logistic fitting function and calculation of 95% upper and lower levels of confidence (Figure 7) resulted in the EC_{50} value. An EC_{50} value of 102.77 h × mL/(min × cm^2) + 8.56/−6.45 was found for formaldehyde in this test setup.

Ozone was continuously generated from UV irradiation of clean air in concentrations between 0.02 and 17.5 ppm and applied to the formaldehyde exposures using a comparable procedure. Results are shown in Figure 8. An EC_{50} value of 29.63 h × mL/(min × cm^2) + 28.08/−9.36 was found for ozone in this test setup.

Variations (standard deviations) in the results between four technical replicates of the exposure groups (formaldehyde or ozone, clean air exposures, or nonexposure controls) were always less than 15%.

Table 1 and Figure 9 compare the results of the improved exposure procedure with the results from the prevalidation study. In both studies, the relative toxicity of ozone was found to be higher than formaldehyde toxicity, although the EC_{50} values from the improved procedure were smaller in both cases by a factor of 28% (ozone) and 27% (formaldehyde).

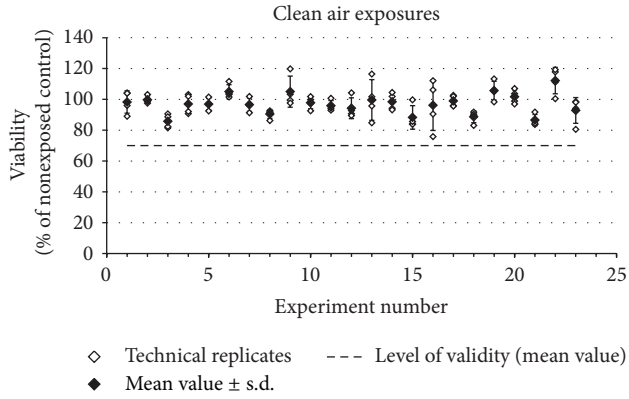
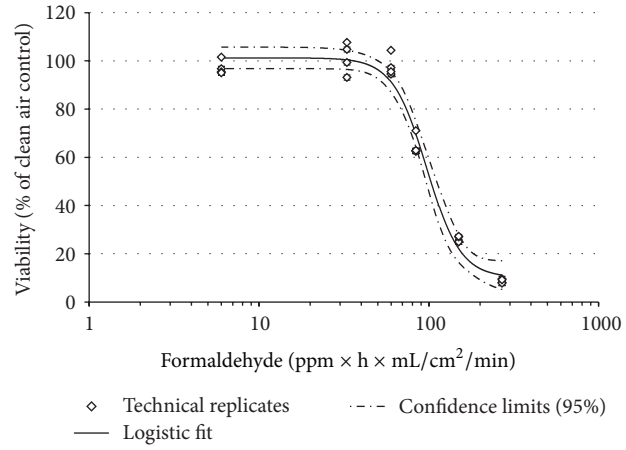


Figure 6: Results of clean air exposures using the ExpoCube (single A549 monolayer cultures with 1 cm^2 growth surface). Results were calculated as percentages of control values with respect to nonexposed controls from WST-1 readings from cultures of the same multiwell plate. The "level of validity" was derived from the prevalidation study [15].



$y = A2 + (A1 - A2)/(1 + (x/x0)^p)$, $r^2 = 0.98$

	Value	Standard error
$A1$	101.24	2.14
$A2$	10.09	3.42
$x0$	97.07	4.16
p	4.34	0.63

Figure 7: Results from exposures to formaldehyde (single A549 monolayer cultures with 1 cm^2 growth surface). Results were calculated as percentages of control values from WST-1 readings with respect to clean air controls within the same multiwell plate.

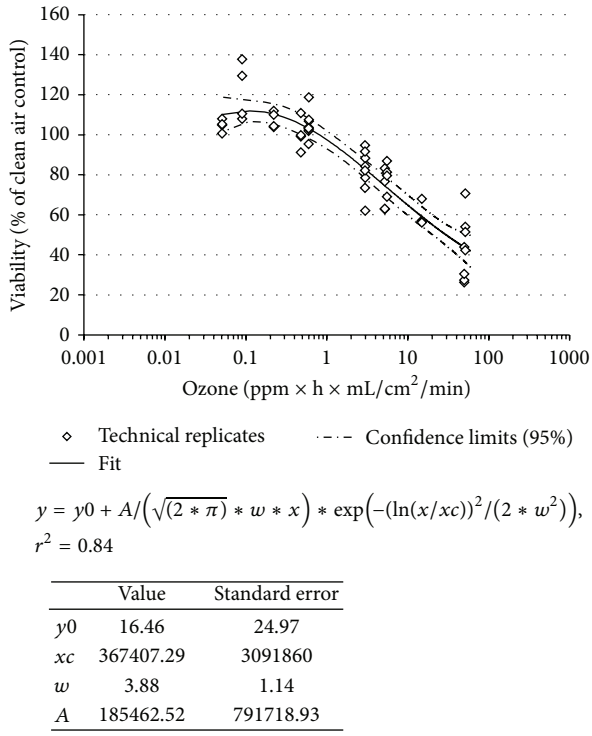
4. Discussion

An improved device-based procedure for cell-based studies on the biological action of airborne substances using the air-liquid interphase (ALI) culture setup was developed. In the light of the results from a meaningful prevalidation study on toxicity testing of chemical gases *in vitro*, several common drawbacks of current procedures and technologies have been identified which represent definite problems for establishing

Table 1: Comparison of EC_{50} values as results from exposures with the improved ALI exposure procedure (ExpoCube) and from the prevalidation study (four partner labs).

	EC_{50} [ppm×h×mL/min×cm^2]				
	ExpoCube			Prevalidation study*	
		UCL	LCL		s.d.
Formaldehyde	102.77	8.56	6.45	140.38	71.55
Ozone	29.63	28.08	9.36	41.13	25.33

UCL: upper confidence limit (95%) from fitting.
LCL: lower confidence limit (95%) from fitting.
s.d.: standard deviation (partner labs from round robin study).
*See [15].



	Value	Standard error
$y0$	16.46	24.97
xc	367407.29	3091860
w	3.88	1.14
A	185462.52	791718.93

Figure 8: Results from exposures to ozone (single A549 monolayer cultures with 1 cm^2 growth surface). Results were calculated as percentages of control values from WST-1 readings with respect to clean air controls within the same multiwell plate.

this promising cell-based *in vitro* technology as a "routine" standard. These drawbacks include the cellular environment during exposure, the unintended exposure route via the culture medium, the process workflow, cellular stress and contamination by the processing of the cultures, the complexity and limited mobility of the setup, low throughput and level of automation, limited observation of cellular changes during exposure, and separate handling of exposure groups and controls.

The principle of the construction of the ExpoCube combines the benefits of the two classical, different flow setups (stagnation flow setup and "incubator-type" setup) for the first time. The better defined and easier to control flow situation from the stagnation flow setup and the benefit of

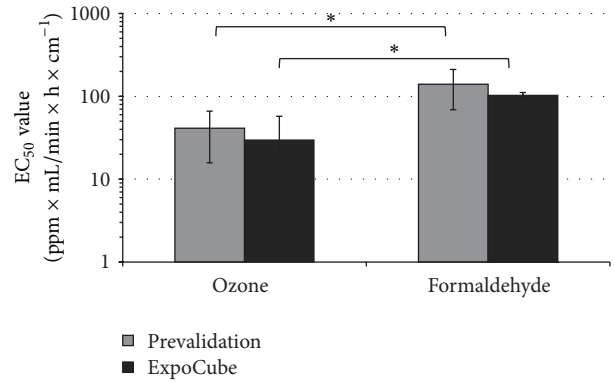


Figure 9: Comparison of results from the prevalidation study and ExpoCube exposures of A549 cells to ozone and formaldehyde. Prevalidation data: s.d., t-test (unpaired, two-sided); ExpoCube data: 95% confidence limits, $^*P < 0.05$.

the ease of use from the "incubator-type" setup are united in one solution for the exposure of ALI cultures on commercial supports in commercial multiwell plates. At the same time, the construction guarantees the complete separation of exposure airflows and the culture media compartment. These improvements have fundamental consequences for the application of the ALI technology in inhalation-related sciences. On the one hand, the process workflow is transformed into a completely integrated design based on commercial multiwell plate use throughout (Figure 1). Cultures in plates can easily be transferred to the exposure situation without a change of culture medium or other manipulations of the cellular environment. Hence, occasional microscopic inspection as an intermittent step during the exposure is possible as well as reexposure following an incubation period after a first exposure in the cell culture incubator. Since the culture medium does not have to be changed throughout this procedure, it is possible to study cellular secretions such as interleukins or chemokines throughout the whole process. This is a fundamental requirement when conducting reproducible and relevant experimental repeated-dose exposure designs, offering the possibility of shifting cell-based assays from investigations of acute to more chronic exposure situations. From the point of view of laboratory organisation and time and money resources, this is a clear improvement, also because the necessary handling steps and sources of contamination are clearly reduced and consumables can be used without the need for sterilisation procedures.

Moreover, additional pitfalls can be prevented by using commercial consumables instead of custom-made housings. Rach et al. [30] detected biological incompatibilities from custom-made metal housings in a specific stagnation flow ALI exposure device. Such a common problem in custom devices is fundamentally impossible in the improved procedure. Here, the medium compartment of the setup (i.e., the multiwell plate), as the most wetted part in the exposure device, is plastic cell culture-compatible consumable labware that has been tested for biocompatibility by the manufacturer.

The entire separation of the culture medium and test atmosphere exposure compartment of the setup is an aspect

that has been completely disregarded in ALI exposure technology up until now. When setups in the "incubator-type" alignment [26, 27] are used, but also in stagnation flow setups [25, 31], the test atmosphere can readily diffuse into the culture medium and will therefore readily react with culture medium components, which include many reactive substances such as glutathione and others. Hence, the cell exposure is not only by way of air-liquid cell exposure but also by a route via the culture medium, which is normally not intended in these studies. Such technical shortcomings may therefore affect the data substantially.

The compactness of the setup and the arrangement of all technical parts in only two compact devices (ExpoCube and ControlUnit) result in a fundamental mobility and readiness for routine use for the newly developed device. To fulfil the demand for a higher throughput, a grouping of single units (Figure 5) was chosen which might represent good agreement between the possibilities of working in smaller or larger setups. Working in smaller setups is often needed due to the high costs of cell cultures (especially primary cultures and complex multicell-type coculture models), whereas the possibility of conducting studies including a high number of technical replicates or different exposure groups is urgently needed for routine toxicity screening of inhalable compounds. Both types of study are possible this way.

To get an insight into cellular changes during exposure with the ExpoCube, two strategies can be used. On the one hand, the exposure can be occasionally stopped, the plate can be taken out of the ExpoCube, cells can be inspected by any multiwell plate-based standard method in the lab, and afterwards the exposure can be restarted by returning the plate to the exposure device. Since this procedure does not lead to any changes in the cellular environment, it might be a good way of performing an occasional process control, especially during method development. Moreover, cultures can be easily monitored online during exposure by using standard multiwell plate-based laboratory methods for fluorescence or luminescence readings. By operating the ExpoCube without a heating plate, the multiwell plate can be positioned on plate-compatible devices such as fluorescence microscopes. Live cell imaging using single cell fluorescence measurements can be carried out adequately during exposure to inhalable substances. Owing to the temperature control of the ExpoCube exposure attachment, this procedure is possible on a standard fluorescence microscope stage without a cell incubator attachment. This is an important step forward for integrating the research on airborne compounds into the broad range of possibilities that have already been developed for cell-based methods using water-soluble substances. Thus, fluorescence- and luminescence-based high-content screening approaches as well as reporter gene assays [32–34] can now be applied to the study of the toxicological and pharmacological action of airborne and inhalable substances, too.

In a first set of experiments, the multiwell plate-based exposure process was examined by exposing A549 cells to clean air, formaldehyde, and ozone. These substances had been tested in a prevalidation study on *in vitro* toxicity testing using the basic ALI setup. Therefore, those results can be taken as a reference for comparison. The basic setup of plate layout and exposure groups already takes into account the results and demands from that study. It had been shown that a nonexposed control group is important to confirm the validity of each experiment. An additional exposure control (usually clean air) should be run parallel with the test group in each experiment. These requirements can now be completely realised using the "all-in-one-plate" concept by grouping cultures in one single multiwell plate. Hence, the fundamental requirements of high reproducibility and relevance of results are satisfied.

Results from exposures to clean air (Figure 6) showed that no damaging cellular stress was induced during exposure. During prevalidation, a limit of validity at 70 % viability (clean exposures versus nonexposed controls) had been defined for the mean of all technical replicates in one exposure experiment. This limit was exceeded by all experiments and even all single technical replicates. The clean air has not been humidified, heated, or enriched with CO_2, which would be the standard procedures to achieve compatibility with culture conditions. Hence, this situation represents some kind of "extreme" condition to document the robustness of the exposure procedure. Nevertheless, it is fundamental for environmental, workplace, or indoor-related cell-based studies as well as for critical inhalable test substances or mixtures that tend to change their physical or chemical state when heated, humidified, or modified by CO_2 mixing (reactive volatile organic chemicals, particle or droplet aerosols, and others).

Exposures to formaldehyde and ozone resulted in reproducible dose-response relationships with only small confidence intervals. EC_{50} values were calculated to be compared with results from the recent prevalidation study (Table 1). The comparison shows that the toxicity ranking of formaldehyde and ozone, with a significantly higher toxicity of ozone, was met. Moreover, the absolute EC_{50} values were within the intervals determined during prevalidation of the ALI testing procedure. However, both values are more on the lower side of the interval from the prevalidation study, which might indicate a slightly higher sensitivity of the improved system due to CFD-optimised flow and thus more efficient contact between the test atmosphere and the culture.

5. Conclusion

Owing to scientific, economic, and also ethical reasons, there is an increasing intensity in toxicological and pharmacological research to develop *in vitro* methods and cell-based assays to assess the biological action of substances and mixtures. In Europe, the REACH legislation is one of the driving factors that increase the demand for toxicological studies on chemicals significantly [35]. Although some *in vitro* tests have already been set up in OECD testing guidelines (OECD guidelines for the testing of chemicals, testing guidelines 438 (eye irritation) and 439 (skin irritation)), test methods for *in vitro* inhalation-related investigations are completely lacking. Moreover, the toxicological cocktail effect of chemicals is of increasing concern [36]. This research field has almost no prospect of being solved *in vivo* due to the enormous

amount of research that would be necessary. From a more pharmacologic point of view, *in vitro* methods need to be designed such that they can deliver a maximum of information on induced cellular changes and kinetics. For both focuses, the toxicological as well the pharmacological, routine use, reproducibility, and relevance of results are the fundamental requirements of a cell-based method.

Taking into account the developments in *in vitro* inhalation testing from the last 40 years, which started with the work of Voisin et al. [4–7], and the results of a current relevant prevalidation study of the basic method [15, 22], an analysis of common drawbacks and limitations was carried out. Fundamental improvements to the exposure situation with respect to the cellular environment, overall performance, and applicability, operability of online investigations during the cell exposure und applicability in a routine setup were introduced by designing a completely integrated "all-in-one-plate" workflow based on commercial standard multiwell plates for *in vitro* inhalation-related research using a device-based (ExpoCube) procedure. These new facilities also enable the further development of procedures, including repeated dose exposures, which are applicable only in the work with water-soluble substances but up until now were hard to realise in reproducible form with inhalable compounds.

The definite goal should be to set up methods for testing airborne and inhalable compounds which are as applicable as the current testing possibilities of water-soluble substances in culture mediums using submerged culture conditions in cell-based assays. Testing inhalable compounds *in vitro* is still a demanding task for specialists in aerosol science because of both practical and theoretical issues from the biological and fluid dynamics points of view. Some fundamental challenges occurring during these studies have been improved by the new procedure. Furthermore, first exploratory results have shown that the new development is in good agreement with the results from prevalidation.

The application of particle or droplet aerosols in cell-based inhalation testing is one further important field of research. Here, experiments are underway to characterise the improved exposure procedure with respect to fluid dynamics-related issues, particle deposition characteristics, and application in aerosol testing.

In summary, the results show that it was possible to create an improved cell-based *in vitro* method for investigations into the biological action of airborne and inhalable substances for routine use. Thus, this seems to be a further step in the sense of the "3R" principle.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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