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Assessment of the acute inhalation toxicity of airborne particles by exposing cultivated human lung cells at the air-liquid interface

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To whom it may concern,

Please find attached a revised version of our manuscript „*Assessment of the acute inhalation toxicity of airborne particles by exposing cultivated human lung cells at the air-liquid interface*" which we would like to resubmit for publication in *Journal of Video Experiments*.

We thank the editor and the four reviewers for their valuable comments and their time and effort to review our manuscript. We have carefully revised the whole manuscript and implemented all suggested changes.
Revisions in the text are in red with strikethrough for deletions. The 2.75 pages for the video process (highlighted in yellow) as well as the detailed responses to the reviewers' comments are provided in separate files.

We are looking forward to your reply.

On behalf of all co-authors

Yours sincerely,

Amelie Tsoutsoulopoulos

TITLE:

Assessment of the acute inhalation toxicity of airborne particles by exposing cultivated human lung cells at the air-liquid interface

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KEYWORDS:
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SUMMARY:
We present a robust, transferable and predictive in vitro exposure system for the screening and monitoring of airborne particles concerning their acute pulmonary cytotoxicity by exposing cultivated human lung cells at the air-liquid interface (ALI).

ABSTRACT:
Here, we present a specially designed modular in vitro exposure system that enables the homogenous exposure of cultivated human lung cells at the ALI to gases, particles or complex atmospheres (e.g., cigarette smoke), thus providing realistic physiological exposure of the apical surface of the human alveolar region to air. In contrast to sequential exposure models with linear aerosol guidance, the modular design of the radial flow system meets all requirements for the continuous generation and transport of the test atmosphere to the cells, a homogenous distribution and deposition of the particles and the continuous removal of the atmosphere. This exposure method is primarily designed for the exposure of cells to airborne particles, but can be adapted to the exposure of liquid aerosols and highly toxic and aggressive gases depending on the aerosol generation method and the material of the exposure modules.

Within the framework of a recently completed validation study, this exposure system was proven as a transferable, reproducible and predictive screening method for the qualitative assessment of the acute pulmonary cytotoxicity of airborne particles, thereby potentially reducing or replacing animal experiments that would normally provide this toxicological assessment.

INTRODUCTION:
Inhalation of toxic airborne particles is a public health concern, leading to a multitude of health risks worldwide and many millions of deaths annually[1,2]. Climate change, the ongoing industrial development and the rising demand for energy, agricultural and consumer products have contributed to the increase of pulmonary diseases over the last years[3–6]. Knowledge and evaluation of inhalable substances regarding their acute inhalation toxicity provide the basis for hazard assessment and risk management, but this information is still lacking for a wide range of these substances[7,8]. Since 2006, the EU chemical legislation REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) requires that already existing and newly introduced products undergo a toxicological characterization including the inhalation route before being placed on the market. Therefore, REACH focuses on alternative and animal-free methods, the implementation of the "3R" principle (Replacement, Refinement, and Reduction of animal experiments) and the use of appropriate in vitro models[9]. In recent years, many different and

adequate non-animal inhalation toxicity testing models (e.g., in vitro cell cultures, lung-on-a-chip models, precision cut lung slices (PCLS)) have been developed in order to assess the acute inhalation toxicity of airborne particles[5,7,10,11]. In terms of in vitro cell culture models, cultivated cells can be exposed under submerged conditions or at the ALI (**Figure 1**). However, the validity of submerged exposure studies is limited with regard to the evaluation of the toxicity of airborne compounds especially particles. Submerged exposure techniques do not correspond to the human in vivo situation; the cell culture medium covering the cells may affect the physico-chemical properties and thus, the toxic properties of a test substance[12,13]. ALI in vitro inhalation models allow the direct exposure of cells to the test substances without interference of the cell culture medium with test particles, thus, mimicking human exposure with higher physiological and biological similarity than submerged exposures[12,14].

For regulatory processes such as REACH, however, only animal models are available in the field of acute inhalation toxicology, as no alternative in vitro methods have been sufficiently validated and officially accepted so far[14]. For this purpose, test models have to be validated according to the requirements of the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) principles on test validity[15].

A former pre-validation study and a recently completed validation study successfully demonstrated the application area of the CULTEX RFS exposure system and its transferability, stability, and reproducibility[13]. This exposure system is an in vitro cell-based exposure system that enables homogenous exposure of cells to gases, particles or complex atmospheres (e.g., cigarette smoke) at the ALI due to its radial aerosol distribution concept and the conduction of the test aerosol in a continuous flow over the cells[16]. The basic module of this radial flow system consists of the inlet adapter, the aerosol guiding module with a radial aerosol distribution, the sampling and socket module, and a locking module with a hand wheel (**Figure 2**). The generated particles reach the cells via the inlet adapter and the aerosol guiding module and are deposited on the cell culture inserts, which are located in the three radially arranged exposure chambers of the sampling module. The aerosol guiding module as well as the sampling module can be heated by connecting to an external water bath[17].

Within the framework of both studies, A549 cells were used for all exposure experiments. The cell line A549 is a human immortalized epithelial cell line that is very well-characterized and has been used as an in vitro model for type II alveolar epithelial cells in numerous toxicological studies. The cells are characterized by lamellar bodies, the production of surfactant and a number of inflammation-relevant factors[18]. They also show properties of bronchial epithelial cells due to their mucus production[19]. Moreover, they can be cultured at the ALI. Although this cell line is deficient in building cell-cell contacts, the cultivation of these cells is much more convenient, less cost expensive and results derived thereof are donor-independent compared to primary cells[20].

A549 cells were seeded in 6-well cell culture inserts (PET membrane, 4.67 cm^2, pore size 0.4 mm) with a density of 3.0 x 10^5 cells per insert and cultivated for 24 h under submerged conditions. Cells were then exposed in three independent laboratories to clean air and three

different exposure doses (25, 50, and 100 µg/cm²) of 20 test substances at the ALI. The exposure dose is correlated to the deposition time resulting in a constant particle rate of 25 µg/cm², 50 µg/cm² and 100 µg/cm² onto the cells after 15, 30 or 60 min, respectively. The deposited particles, however, were not washed off after deposition, but remained on the cells for 24 h. The deposition times of the particles were therefore 15, 30 and 60 min, but the exposure of the cells lasted for 24 h in total. The deposition rate of the test substances was determined in preliminary experiments according to previous methods[17].

Cell viability as an indicator of toxicity was assessed 24 h after particle deposition using a cell viability assay. Special focus was set on the quality of clean air controls, the optimization and refinement of the exposure protocol, the intra- and inter-laboratory reproducibility and the establishment of a prediction model (PM). Substances that led to a decrease of cell viability below 50% (PM 50%) or 75% (PM 75%) at any of the three exposure doses were considered to exert an acute inhalation hazard. Results were then compared to existing in vivo data (based on at least one reliable study according to OECD test guideline (TG) 403 or TG 436[21,22]), leading to an overall concordance of 85%, with a specificity of 83% and a sensitivity of 88%[23].

Besides the measurement of cell viability, other endpoints such as cytokine release, examination of the cell lysate or membrane integrity via LDH assay can be assessed but were not required for the validation study. Thus, the exposure system (e.g., CULTEX RFS) was proven as a predictive screening system for the qualitative assessment of the acute inhalation toxicity of the tested airborne particles, representing a promising alternative method to animal testing. The following protocol is recommended for exposure experiments to airborne particles using this exposure system.

PROTOCOL:

NOTE: The protocol of one exposure experiment covers a period of three days.

Day 1

1. General preparations and cultivation of cells

NOTE: The human lung adenocarcinoma epithelial cell line A549 was used for exposure experiments. Cells must be handled under sterile conditions. Other cell lines that are suitable for cultivation at the ALI can be used.

1.1. Prepare the growth medium (Dulbecco's Minimum Essential Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 5 µg/mL gentamycin) and the exposure medium (DMEM, supplemented with 5 µg/mL gentamycin and a final HEPES concentration of 100 mM).

1.2. Culture A549 cells in growth medium at 37 °C in a humidified atmosphere containing 5% CO_2.

1.3. Cultivate cells in cell culture flask of 75 cm² (T-75) in 14 mL of growth medium until a confluence of 80-90% before splitting (every 2-3 days) and a passage of 35.

2. Trypsinization of cells

2.1. Temper phosphate buffered saline (PBS) and growth medium at 37 °C and the trypsin/EDTA (0.05%/ 0.02%) solution at room temperature.

2.2. Aspirate the cell culture medium from the cell culture flask and wash the cells carefully with 8 mL of pre-heated PBS.

2.3. Remove the PBS, add 2 mL of the trypsin/EDTA (0.05%/ 0.02%) solution to the cells and incubate for maximal 6 min. in the incubator at 37 °C. Control the detachment process under the microscope.

2.4. Neutralize the trypsin activity by adding 8 mL pre-heated growth medium, detach the cells by gently tapping sideways on the flask and resuspend the cells by repeated pipetting up and down.

2.5. Transfer the cell suspension to a 50 mL tube. Determine the cell number for further procedure (e.g., seeding of cells, passage of cells).

3. Determination of cell number

NOTE: Cell concentration was determined using a cell counter or counting chambers.

3.1. Dilute 100 µL of the cell culture suspension in a cup filled with 10 mL of isotonic solution. Tilt the cup slowly without shaking.

3.2. Determine number of viable cells/mL and cell viability based on the cell-specific measurement parameters of A549 cells.

4. Seeding of cells onto microporous membranes in cell culture inserts

NOTE: The exposure system is equipped with special adapters to enable the use of commercial inserts from different suppliers and of different sizes. For these exposure experiments, 6-well plates and the corresponding cell culture inserts were used. All working steps have to be done under sterile conditions.

4.1. Calculate the suspension volume and the required number of cell culture inserts (three cell culture inserts as incubator controls and cell culture inserts for clean air and particle exposure) and cell culture plates. Provide pre-heated growth medium under sterile cell culture conditions (laminar flow).

4.2. Before seeding the cells, add 2.5 mL of tempered growth medium to each well of a 6-well plate. Place the cell culture inserts without cells carefully inside the wells and add 1 mL of growth medium to every cell culture insert. Incubate the 6-well plates for at least 30 min in the incubator (37 °C, 5% CO_2).

4.3. Prepare a sufficiently high volume of the cell suspension with a cell concentration of 3.0 x 10^5 cells/mL.

4.4. After 30 min, aspirate the medium within the cell culture inserts and seed 1 mL of A549 cells with a density of 3.0 x 10^5 cells/mL in each cell culture insert. Distribute the cell suspension by gentle rocking.

4.5. Incubate the cell culture inserts with the cell suspension for 24 h (37 °C and 5% CO_2).

5. Pressing of test substances

NOTE: Test substances were pressed into powder cakes using a fully controllable hydraulic press. The press package can apply a maximum force of 18 kN, which is displayed as the current oil pressure (in bar) of the press package. Press conditions (pressing pressure, time of pressing) of unknown test substances have to be established and characterized in preliminary tests. Depending on the press properties of a substance, different pressing parameters and kinds of pressing plunger can be used.

CAUTION: Wear protective equipment when pressing toxic or dangerous substances.

5.1. Set the pressing time via the time control on the front side of the press.

5.2. Open the compressed air supply at the compressed air valve. Set the compressed air pressure to approximately 2 bar (indicated by a pressure gauge on the front side) using the pressure regulator on the front side of the press or on the compressed air valve of the compressed air supply. Pull out the drawer, press the **Press** button and read the pressing pressure on the digital pressure switch.

5.2.1. Readjust the pressure at the pressure regulator if the pressure is too high or too low.

5.3. Assembly the substance container and ensure that the glass cylinder is correctly centered (**Supplementary Figure 1**). Fill the substance container with a small amount of the test substance. Insert the plunger into the substance container and turn it slightly back and forth to evenly distribute the powder in the container.

5.4. Place the substance container with the plunger in the drawer and press the **Press** button. The hydraulic piston of the press moves onto the plunger and exerts a pressure on the test substance for the set pressing time. Open the drawer and remove the plunger.

5.5. Repeat steps 5.3 and 5.4 until the substance container is at least half full.

5.6. After completion of the pressing work, remove the substance container from the drawer and turn it upside down to remove loose and deposited particles.

5.7. If the substance container is not needed at the same day, close the substance container with parafilm in order to prevent the test substance from drying out or absorbing moisture.

Day 2

6. Assembly of the exposure system and connecting the peripheral equipment

NOTE: A more detailed view is provided in **Figure 3**, **Supplementary Figure 2** and **Supplementary Figure 3**. Assemble both modules and the aerosol generator according to the manufacturer's instructions.

6.1. Place the exposure system on a solid and even surface, with the water supply facing forward. Connect the mass flow controllers with the aerosol generator and a three-necked bottle connected to the module for clean air exposure.

6.2. Connect the flow controller and the vacuum pump. Connect the tubes from the flow controllers with the tube connector on the attachments of the aerosol guiding module. Connect the tubes on the other side of the flow controllers with the vacuum pump. Make sure that the flow is going from the module through the flow controllers to the vacuum pump.

6.3. Connect the water bath with the heating water supply. The water supply is going from the water bath to the water inlet on the aerosol guiding module. Connect the water outlet of the aerosol guiding module with the water inlet of the sampling module. Close the circle with a connection from the water outlet of the sampling module to the water bath.

6.4. Place the aerosol generator including the elutriator close to the exposure module and connect the excess lines of the elutriator and the exposure and clean air module with large micro filters, and the suction of the exposure chambers with small micro filters (e.g., disposable filters). The elutriator serves as a reservoir for the generated particulate atmosphere and retains particles bigger than approx. 7 µm, whereas smaller particles are transported to the exposure module.

6.5. Connect the computer used to control the aerosol generation to the USB port of the aerosol generator top via a USB cable and the power supply to the power supply port. Connect the AC power plug of the power supply unit to a socket (220-240 V).

6.6. Connect the pipes for the medium supply and removal with two pumps. Instead of using a pump for the medium supply, the medium can also be filled manually.

7. Preparation for clean air and particle exposure

7.1. Turn on the vacuum pump, the flow controllers and the water bath (37 °C) for a warm-up period of at least 30 min.

7.2. Open the compressed air supply. Set the mass flow controllers to 8 L/min for the supply line to the aerosol generator and to 3 L/min for the supply line to the three-necked bottle. Close the tabs of the mass flow controllers.

NOTE: These values may vary depending on the characteristics of the test substance.

7.3. Adjust the flow controllers via the computer to regulate the module flow (1.5 L/min) and the chamber suction (30 mL/min).

8. Leakage test of the radial flow system

NOTE: The leakage check must be performed under vacuum and for both modules (exposure and clean air module) in order to ensure that the module has been reassembled properly.

8.1. Remove the inlet adapter and the condensate reflector from the aerosol guiding module. Close the three aerosol feeding bores in the aerosol guiding module with plugs and the medium supply connections at the sampling module with dummy flaps.

8.2. Connect the vacuum lines without the filter with the tube connector of the aerosol guiding module. Close the module by using the hand wheel and measure the value of the flow controllers. The values should decrease some minutes after closing below 5 mL/min.

8.3. After the impermeability check, remove all plugs and dummy flaps, insert the inlet adapter and the condensate reflector into the aerosol guiding module and connect the pipes for medium supply and removal.

9. Aerosol generation

9.1. Start the computer and the software (**Supplementary Figure 4**). Start the aerosol generator software by double clicking on the aerosol generator start button on the desktop of the computer. A message window appears and asks if the settings should be reset or not. Click **Yes** if the software is started for the first time that day. Set the values for **Slide Position** and **Scraper Position** to the default values. Click **No** to keep the values for **Slide Position** and **Scraper Position** or the slide is not in the starting position.

9.2. Screw a substance scraper into the pipe, which is located in the central opening of the aerosol generator top.

NOTE: Depending on the press characteristics, distinct types of substance scraper can be used.

9.3. Use the button **Homing Mode** if the substance scraper is not in the lowest position.

9.4. Place the substance container with the pressed test material upside down over the substance scraper. Ensure that the glass of the substance container faces the front. Make sure that the two holes in the substance container fit onto the two pins of the aerosol generator top. Place the locking plate in the slot over the substance container and tighten the black screw.

9.5. Change the values for **Feed** (0.24 to 20 mm/h) and **Rotation** (1 to 800 revs/h) to the desired settings. The particle concentration can be modified by increasing or decreasing the **Feed** value or the carrier gas flow rate.

9.6. Use the downward arrows to push down the slide with the substance container until the substance scraper is near the pressed substance.

9.7. Open the compressed air supply to the aerosol generator with a tap of the mass flow controller and start the aerosol generation by clicking on the **Start** button. Set the **Feed** rate to 15-20 mm/h to avoid long waiting times.

9.8. Control the correct particle generation by observing the fine dust cloud with a small flashlight (positioned from below behind the glass tube of the Elutriator). Change the value for **Feed** back to the desired settings when the first aerosol vapor reaches continuously the Elutriator and click on the **Stop** button.

10. Exposure experiments

10.1. Start the medium supply with pre-heated exposure medium and fill the sampling modules until the downpipes are covered while the module is open. Use the medium pump or fill the medium manually (25 mL per individual exposure chamber).

10.2. Insert blind cell culture inserts (inserts without cells) into the exposure module. Pump the exposure medium down until the downpipes are covered with medium and the lower side of the inserts are in contact with medium.

10.3. Start the aerosol generator, close the exposure module and connect the exposure module to the exposure module outlet of the aerosol generator. Give the aerosol generator a lead time of at least 20-30 min before exposures are started in order to enable a stable generation of particles.

10.4. Prepare the post-incubation plates for the incubator controls and the exposed cell culture inserts during the lead time. Add 1.5 mL of growth medium per well and incubate the plates in the incubator (37 °C, 5% CO_2).

10.5. After the lead time, seal the exposure module outlet of the elutriator with a rubber plug and remove the blind inserts. Refill the exposure medium (using the pump or manually) until the downpipes are covered with medium.

10.6. Remove the cell culture inserts from the 6-well plates with the help of a tweezer. Pour the growth medium carefully from the cell culture inserts off by toppling the inserts and aspirate and discard the residual liquid using a pipette. Place the inserts in the exposure chambers of both modules, the exposure and clean air module.

10.7. Close the modules and start the exposure experiments by connecting the exposure module to the exposure module outlet of the aerosol generator and the clean air module to the carrier gas supply simultaneously.

NOTE: The particle concentration can be modified by increasing/decreasing the **Feed** value, the carrier gas flow rate or the time of exposure.

10.8. Disconnect the exposure and clean air modules after completion of the experiment and seal the exposure module outlet.

10.9. Stop the compressed air supply and the aerosol generator by clicking on the **Stop** button.

10.10. Open the exposure and clean air module and transfer the cell culture inserts to the prepared post-incubation plates using a tweezer. Incubate the 6-well plates for 24 h (37 °C, 5% CO_2) at the ALI.

NOTE: Repeat steps 10.5 -10.10 if further exposure experiments are planned.

10.11. Lift the cell culture inserts, that are used as incubator controls to the ALI under the same conditions as the exposed cell culture inserts and incubate them for 24 h (37 °C, 5% CO_2) at the ALI.

10.12. Use the button **Homing Mode** to remove the substance container. Close the aerosol generator software by clicking on the **X** in the upper right-hand corner and turn off the computer.

10.13. After completion of all exposure experiments, clean the aerosol generator and both exposure modules. Close the substance container with parafilm if the test substance will be further used within the next days.

Day 3

11. Cell viability

NOTE: Cell viability was determined 24 h after particle deposition by measuring the mitochondrial activity using the WST-1 assay. The assay was performed according to the manufacturer's protocol. Cell viability can also be determined by using other cell viability tests (e.g., XTT).

11.1. Temper growth medium at 37 °C and thaw the WST-1 solution protected from light. Prepare an appropriate number of new 6-well plates with 2.5 mL growth medium per well and incubate the plates in the incubator.

11.2. Prepare the WST-1 dilution by diluting a sufficient amount of WST-1 1:7 in growth medium

11.3. Insert the cell culture inserts 24 h after exposure in the new prepared 6-well plates. Add 1 mL of the fresh-prepared WST-1 solution to each cell culture insert. Rock the plates carefully in order to distribute the solution homogenously on the cells. Incubate the 6-well plates with the cell culture inserts for 1 h (37 °C, 5% CO_2).

11.4. Transfer 100 µL of the supernatant in triplicates from each 6-well to a 96-well plate. Measure the absorbance at 450 nm with a reference wavelength of 650 nm using a microplate reader.

12. Statistics

12.1. Normalize the cell viability of the individual incubator controls to 100%.

12.2. Express the viability of the exposed cells in relation to the individual incubator controls. Cytotoxicity of test substances was compared to the respective incubator controls and used as an indicator of toxicity.

REPRESENTATIVE RESULTS:

The CULTEX RFS is a specially designed modular in vitro exposure system that enables the direct and homogenous exposure of cells at the ALI. Within a former pre-validation study, the general applicability of this exposure system and its transferability, stability and reproducibility were successfully demonstrated. In a recent research project funded by the German Federal Ministry of Education and Research, the exposure system was successfully validated and established as a prediction model (PM) for acute inhalation hazards of the tested compounds. As the quality of the clean air controls turned out to be a critical parameter during the pre-validation study, several protocol and method optimizations (e.g., change of cell culture inserts, stabilization of the pH of the exposure medium by increasing the HEPES concentration to 100 mM) were implemented at the beginning of the validation study, leading to highly stable and reproducible results and a substantial improvement of clean air viability data across all three laboratories (**Figure 4**). A549 cells were then exposed at the ALI to three different exposure doses (25, 50 and 100 µg/cm²) of 20 pre-selected and coded test substances in the three independent laboratories, and cytotoxicity (used as an indicator of toxicity) was compared to the respective

incubator controls. Thirteen coded substances were thereby tested in triplicates, seven coded substances as single experiments. Test substances were considered to exert an acute inhalation hazard when cell viability decreased below 50% (PM 50%) or 75% (PM 75%).

As shown exemplarily in **Figure 5**, exposure of A549 cells to different test substances exhibited no, medium or a strong toxicity. As all experiments were conducted independently in three laboratories, data were analyzed regarding the reproducibility within and between the laboratories and the predictivity of the exposure system. Depending on the applied PM (PM 50% or PM 75%), the within-laboratory and the between-laboratory reproducibility ranged from 90-100%, demonstrating the robustness and transferability of this method. As all tested substances had relevant available in vivo reference data (based on at least one reliable study according to OECD TG 403 or TG 436 using a traditional LC_{50} protocol and a concentration x time (C x t) protocol), comparison of the in vivo and in vitro data revealed an overall concordance of 85% (17/20) with a specificity of 83% (10/12) and a sensitivity of 85% (7/8) (**Table 1**). Only two substances were classified as falsely positive and one as falsely negative.

In summary, our results of the validation study present a transferable, reproducible and predictive screening method for the qualitative assessment of the acute pulmonary cytotoxicity of the selected airborne particles.

FIGURE AND TABLE LEGENDS:

Figure 1: Exposure of cells at the ALI or under submerged conditions. A549 cells can be either exposed with a test substance (blue arrows and dots) at the ALI (left) through an inlet of the exposure system or with the test substance diluted in exposure medium (blue dots) creating submerse conditions (right). Red dotted lines represent the fill levels of exposure medium (bright red) in the respective experimental setup. This figure has been modified from Tsoutsoulopoulos et al.[24].

Figure 2: The exposure module. Schematic overview of the basic module of the radial flow system consisting of the inlet adapter, the aerosol guiding module, the sampling and socket module and a locking module with a hand wheel. This figure has been modified from Aufderheide et al.[17].

Figure 3: Overview of the exposure system. The components are the two exposure modules for clean air and particle exposure, the aerosol generator including the elutriator and corresponding control unit, and the medium pumps. This figure has been modified from Aufderheide et al.[17].

Figure 4: Clean air viability data after optimization of the test method. Exposure of cells to clean air led to no decrease of cell viability over time, leading to a substantial improvement of clean air viability data compared to the pre-validation study. All clean air controls were pooled for each laboratory (Lab 1-3) and exposure time (n = 46 per laboratory and point in time). Data

are displayed as boxplots with a median line and the range indicated by whiskers. This figure has been modified from Tsoutsoulopoulos et al.[23].

Figure 5: Exposure of A549 cells to different test substances.
(**A**) Exposure of A549 cells to tungsten(IV) carbide showed no decrease of cell viability for the three different exposure doses and appeared to be non-toxic (n = 3 per laboratory and exposure dose). (**B**) Exposure of cells to tetrabromophthalic anhydride resulted for all laboratories in a moderate toxicity presenting a good dose response curve (n = 1 per laboratory and exposure dose). (**C**) Zinc dimethyldithiocarbamate exhibited a strong toxicity, leading to a decreased cell viability already after a deposited dose of 25 µg/cm² (n = 3 per laboratory and exposure dose). Error bars represent standard deviations. This figure has been modified from Tsoutsoulopoulos et al.[23].

Table 1: Accordance between in vivo and in vitro results. Out of 20 substances, 10 substances were classified as correctly negative, and seven substances correctly as positive, leading to a concordance of 85% (17/20). This table has been modified from Tsoutsoulopoulos et al.[23]. (Test No. 403: Acute Inhalation Toxicity, Test No. 436: Acute Inhalation Toxicity - Acute Toxic Class Method; Acute Tox. 2 = fatal, Acute Tox. 3 = toxic, Acute Tox. 4 = harmful).

SUPPLEMENTARY FIGURES
Supplementary Figure 1: Assembly of the substance container. Picture taken from CULTEX DG (Dust Generator) User Manual.

Supplementary Figure 2: Aerosol guiding module of the exposure system. A) Top view and B) bottom view of the aerosol guiding module. Picture taken from CULTEX RFS (Radial Flow System) User Manual.

Supplementary Figure 3: The aerosol generator. A) Schematic overview of the aerosol generator, consisting of the aerosol generator top and the Elutriator. Detailed view of B) the aerosol generator top and C) the Elutriator. Picture taken from CULTEX DG (Dust Generator) User Manual.

Supplementary Figure 4: The aerosol generator control software. Picture taken from CULTEX DG (Dust Generator) User Manual.

DISCUSSION:
Many non-animal inhalation toxicity testing models have been developed in recent years in order to gain information about the acute inhalation hazard of inhalable particles and to reduce and replace animal experiments according to the 3R principle[25].

In terms of cell culture models, exposure of cells can be done under submerged conditions or at the ALI. Exposing cells under submerged conditions may affect the physico-chemical properties and thus, the toxic properties of a test substance[12]. In vitro ALI inhalation models, however, mimic the human exposure situation with higher biological and physiological similarity than

submerged exposure and are therefore better suited for analyzing the acute inhalation toxicity of airborne particles. The significance of the CULTEX RFS with respect to other existing exposure modules is not only the exposure of cells under ALI conditions but also the very homogenous distribution and deposition of particles. In contrast to sequential exposure models with linear aerosol guidance, the modular design of this exposure method enables a radial supply line leading to a very homogenous deposition of particles on the cells[17].

The most important point for successful exposure experiments is the stable and congruent quality of the clean air controls. Special attention must be paid that the viability of the clean air controls is not affected over time and as close as possible at 100% compared to the corresponding incubator controls. Factors that play an important role regarding clean air viability are the choice of suitable cell culture inserts, the pH value of the exposure medium, and the composition of the clean air. In terms of cell culture inserts, a good quality and a high density of pores must be guaranteed. This ensures a better medium supply and a higher relative humidity inside the cell culture inserts, protecting the cells from desiccation[26]. By using cell culture inserts with side wall openings, special insert sleeves must be used in order to avoid leakage of test particles through the side wall openings which could lead to a possibly contamination of the exposure medium. A shift of the pH value higher than 8 can already have a toxic effect on the cells and therefore leading to an impairment of cell viability[27]. This occurs especially in prolonged particle deposition times (e.g., 60 min) if the clean air contains less than 5% CO_2 or the HEPES concentration of the exposure medium is too low which has to be avoided.

A critical issue of the protocol is the pressing of the test substances. The test substances have to be sufficiently compressed to a powder cake within the substance container in order to enable a stable particle exposure. Thus, the substances have to be characterized in preliminary experiments regarding their press properties and in order to obtain information about which press plunger, type of scraping blade or feed rates have to be used.

The maximum pressing pressure of the hydraulic press, however, is 10 kN, which represents at the same time the load limit for the glass cylinder and thus, a limitation of the pressing process. The substance container cannot withstand higher pressing forces than 10 kN. A higher pressing force might offer the pressing of crystalline substances and thus, extend the applicability of this press but would require more robust substance containers.

Moreover, this exposure system which is primarily designed for the investigation of airborne particle exposures can be adapted to the exposure of liquid aerosols and highly toxic and aggressive gases depending on the aerosol generation method and the material of the exposure modules. Exchanging the aerosol generator with a membrane nebulizer and using a stainless-steel exposure module enabled the exposure of cells to highly toxic liquid aerosols[24].

A further critical issue is the overload effect. Cell viability can be affected not only by toxicological properties of a test substance but also by the amount of a substance deposited on the cells. This exposure system shows indeed substantial similarity to the physiological

conditions in the human alveolar region but does not contain any clearance mechanisms for removing particles. It is therefore very important that the cell viability is not impaired due to a too large number of particles.

The protocol herein describes the homogenous exposure of cultivated human lung cells at the ALI to airborne particles. The reproducibility, its robustness and transferability make the current exposure system applicable as an in vitro screening method for the qualitative assessment of inhalable particles regarding their acute inhalation toxicity. Because no alternative in vitro methods have been sufficiently validated so far, acute pulmonary toxicity is still being assessed by exposing animals (e.g., whole-body chambers, nose or mouth-only methods). All commonly accepted OECD test guidelines for the acute inhalation toxicity (e.g., TG 403, TG 433 and TG 436) are based on animal models at present[21,22,28,29]. One future direction will be therefore to apply at the Organization for Economic Cooperation and Development (OECD) for the acceptance as an in vitro test guideline for the acute inhalation toxicity.

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DISCLOSURES:

The authors AT, KG, AB, SH, HM, TG, HT and DS have nothing to disclose. The company Cultex Technology GmbH (formerly Cultex Laboratories GmbH) produces instruments (e.g., CULTEX RFS, CULTEX DG) used in this article. NM was an employee of Cultex Laboratories GmbH during this study. OK is an employee of Cultex Technology GmbH (formerly Cultex Laboratories GmbH). The patent PCT/EP2009/007054 for the device is hold by the founder of the Cultex Technology GmbH Prof. Dr. Ulrich Mohr (formerly Cultex Laboratories GmbH).

REFERENCES:

1. Faber, S.C., McCullough, S.D. Through the Looking Glass: In vitro Models for Inhalation Toxicology and Interindividual Variability in the Airway. *Applied In vitro Toxicology*. **4** (2), 115-128 (2018).
2. World Health Organization. Ambient air pollution: a global assessment of exposure and burden of disease. http://apps.who.int/iris/bitstream/handle/10665/250141/9789241511353-eng.pdf?sequence=1. Accessed August 24, 2018 (2016).
3. De Matteis, S. et al. Current and new challenges in occupational lung diseases. *European Respiratory Review*. **26** (146), 1-15 (2017).
4. LANUV Nordrhein-Westfalen. *Gesundheitliche Risiken von Nanomaterialien nach inhalativer Aufnahme*. (2009).
5. Bérubé, K. et al. In vitro Models of Inhalation Toxicity and Disease. The report of a FRAME workshop. *Alternatives To Laboratory Animals*. **37** (1), 89-141 (2009).
6. Lopez, A.D., Murray, C.C. The global burden of disease, 1990-2020. *Nature Medicine*. **4** (11), 1241-1243 (1998).

7. Clippinger, A.J. et al. Alternative approaches for acute inhalation toxicity testing to address global regulatory and non-regulatory data requirements: An international workshop report. *Toxicology In vitro*. **48** (October), 53-70 (2018).
8. Agrawal, M.R., Winder, C. Frequency and Occurrence of LD50 Values for Materials in the Workplace. *Journal Of Applied Toxicology*. **16** (5), 407-422 (1996).
9. Amtsblatt der Europäischen Union. Verordnung (EG) Nr. 1907/2006 des Europäischen Parlaments und des Rates. *Europäische Union*. **860** (2006).
10. Huh, D. et al. Reconstituting Organ-Level Lung Functions on a Chip. *Science*. **328** (5986), 1662-1668 (2010).
11. Fisher, R.L. et al. The Use of Human Lung Slices in Toxicology. *Human and Experimental Toxicology*. **13** (7), 466-471 (1994).
12. Lenz, A-G. et al. Inflammatory and Oxidative Stress Responses of an Alveolar Epithelial Cell Line to Airborne Zinc Oxide Nanoparticles at the Air-Liquid Interface. *Biomed Research International*. **12** (2013).
13. Steinritz, D. et al. Use of the CULTEX Radial Flow System as an in vitro exposure method to assess acute pulmonary toxicity of fine dusts and nanoparticles with special focus on the intra- and inter-laboratory reproducibility. *Chemico-Biological Interactions*. **206** (3), 479-490 (2013).
14. Lacroix, G. et al. Air–Liquid Interface In vitro Models for Respiratory Toxicology Research. *Applied In vitro Toxicology*. **4** (2), 91-106 (2018).
15. Eskes, C., Whelan, M. Validation of Alternative Methods for Toxicity Testing. In: Springer International Publishing. **418** (2016).
16. Rach, J., Budde, J., Möhle, N., Aufderheide, M. Direct exposure at the air-liquid interface: Evaluation of an in vitro approach for simulating inhalation of airborne substances. *Journal Of Applied Toxicology*. **34** (5), 506-515 (2014).
17. Aufderheide, M., Halter, B., Möhle, N., Hochrainer, D. The CULTEX RFS: A comprehensive Technical Approach for the In vitro Exposure of Airway Epithelial Cells to the Particulate Matter at the Air-Liquid Interface. *Biomed Research International*. **15** (2013).
18. Lieber, M., Todaro, G., Smith, B., Szakal, A., Nelson-Rees, W. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *International Journal Of Cancer*. **17** (1), 62-70 (1976).
19. Carterson, A.J. et al. A549 lung epithelial cells grown as three-dimensional aggregates: Alternative tissue culture model for Pseudomonas aeruginosa pathogenesis. *Infection And Immunity*. **73** (2), 1129-1140 (2005).
20. Kim, K.J., Borok, Z., Crandall, E.D. A useful in vitro model for transport studies of alveolar epithelial barrier. *Pharmaceutical Research*. **18**(3), 253-255 (2001).
21. OECD. *Test No. 403: Acute Inhalation Toxicity*. Paris: OECD Publishing (2009).
22. OECD. *Test No. 436: Acute Inhalation Toxicity – Acute Toxic Class Method*. Paris: OECD Publishing (2009).
23. Tsoutsoulopoulos, A. et al. Validation of the CULTEX Radial Flow System for the assessment of the acute inhalation toxicity of airborne particles. *Toxicology In vitro*. **58**, 245-255 (2019).
24. Tsoutsoulopoulos, A. et al. A novel exposure system generating nebulized aerosol of sulfur mustard in comparison to the standard submerse exposure. *Chemico-Biological*

Interactions. **298**, 121-128 (2019).
25. Russell, W.M.S., Burch, R.L. The principles of humane experimental technique. http://altweb.jhsph.edu/pubs/books/humane_exp/het-toc (1959).
26. Tsoutsoulopoulos, A. et al. Optimization of the CULTEX radial flow system for in vitro investigation of lung damaging agents. *Toxicology Letters*. **244**, 28-34 (2016).
27. Osman, J.J., Birch, J., Varley, J. The response of GS-NS0 myeloma cells to pH shifts and pH perturbations. *Biotechnology And Bioengineering*. **75** (1), 63-73 (2001).
28. OECD. Test Guidenline 433: Acute Inhalation Toxicity – Acute Toxic Class Method (2018).
29. OECD. *Guidance Document on Inhalation Toxicity Studies* (2018).

Figure 1

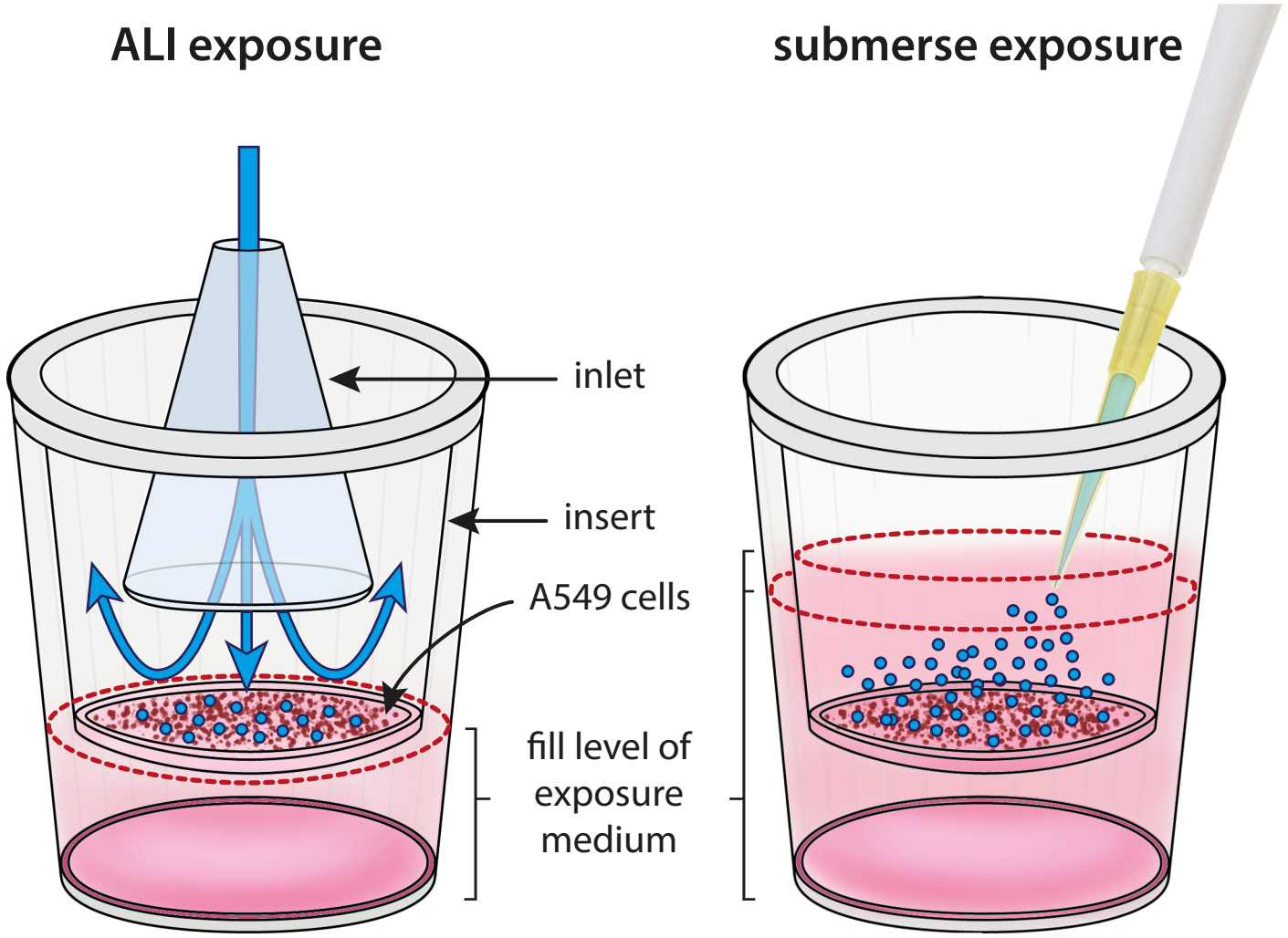

Figure 2

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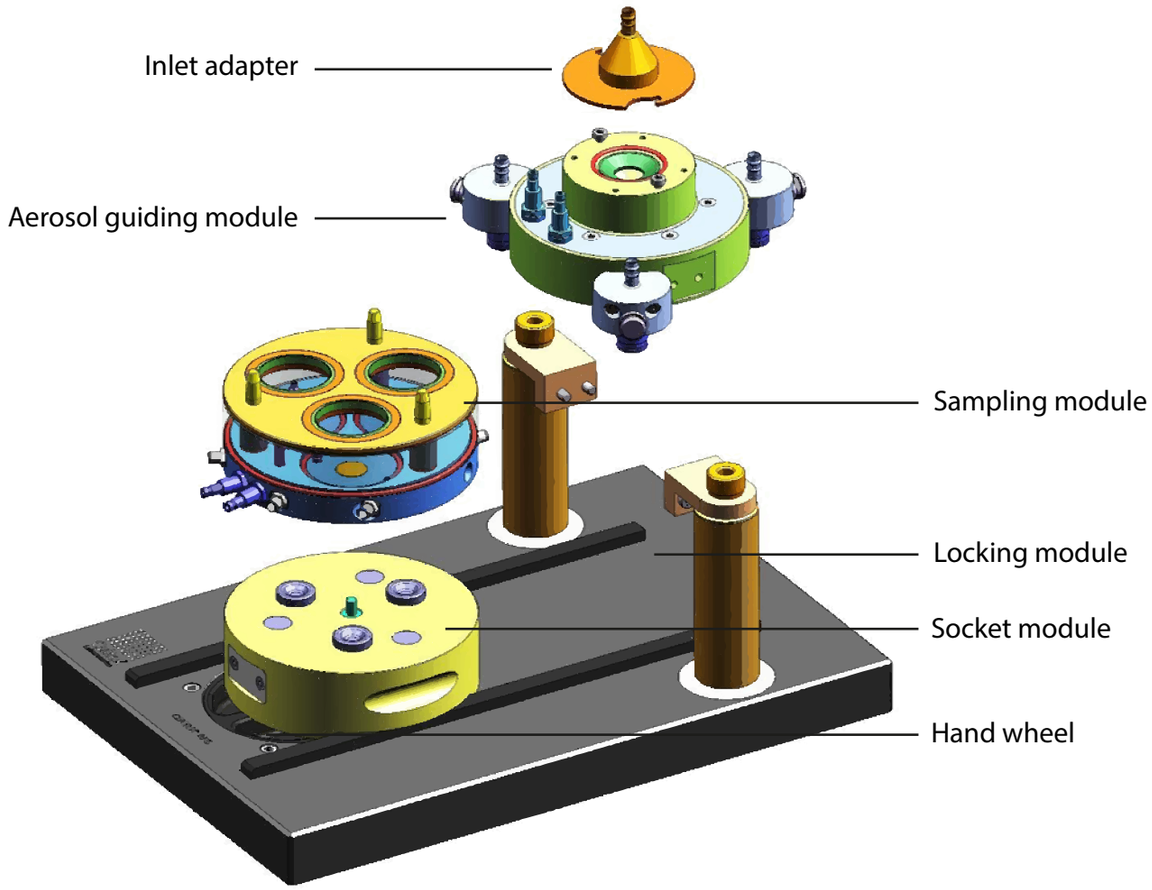

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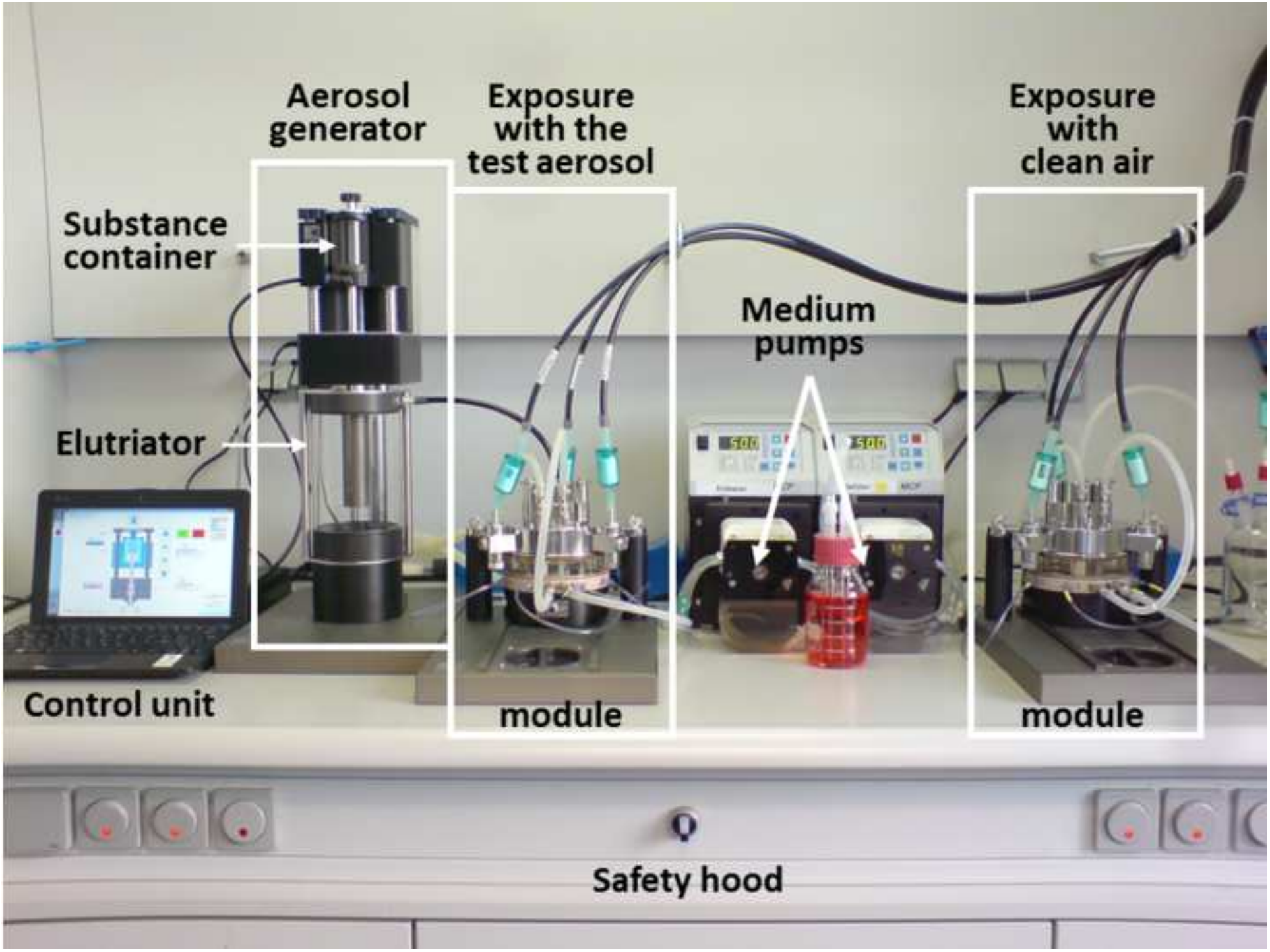

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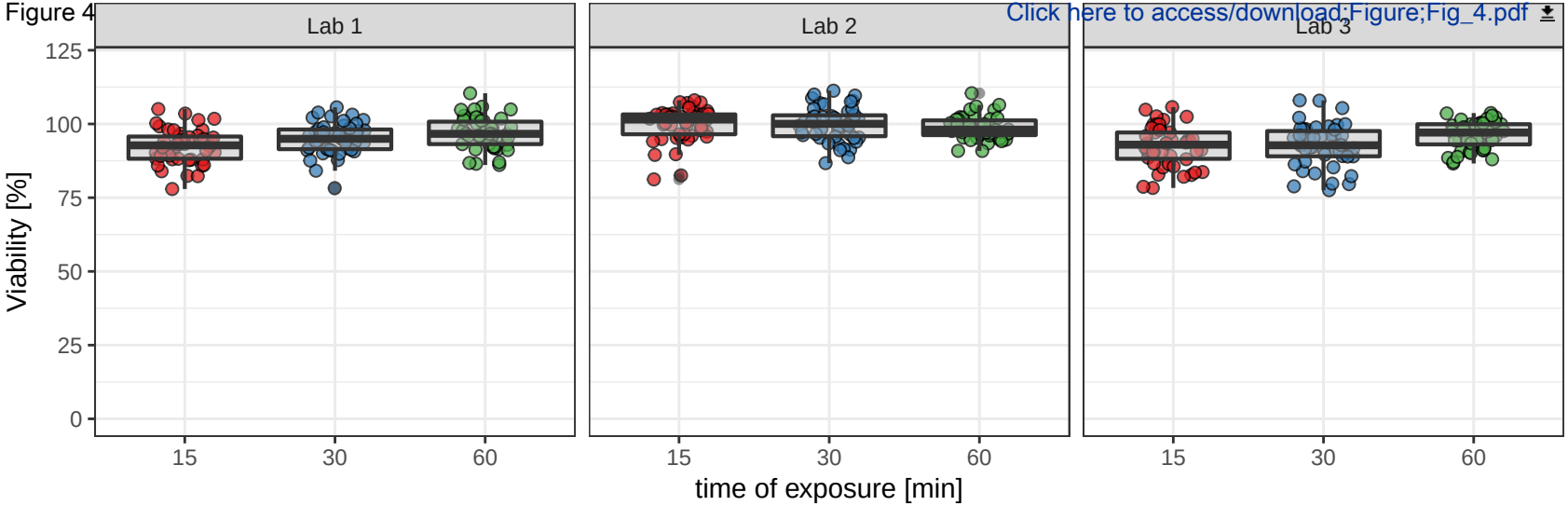

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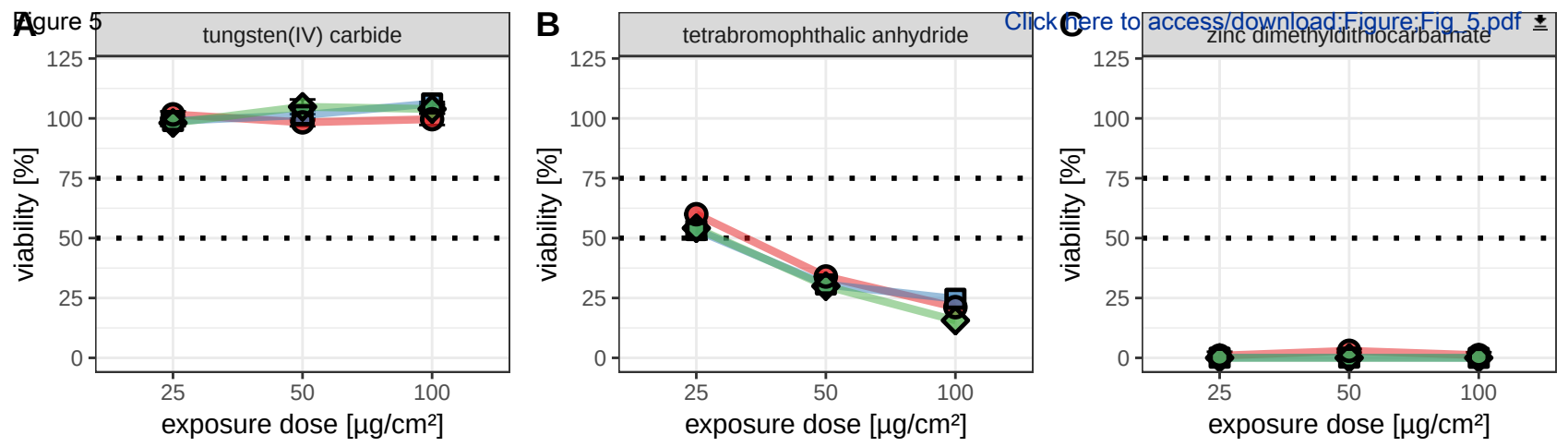

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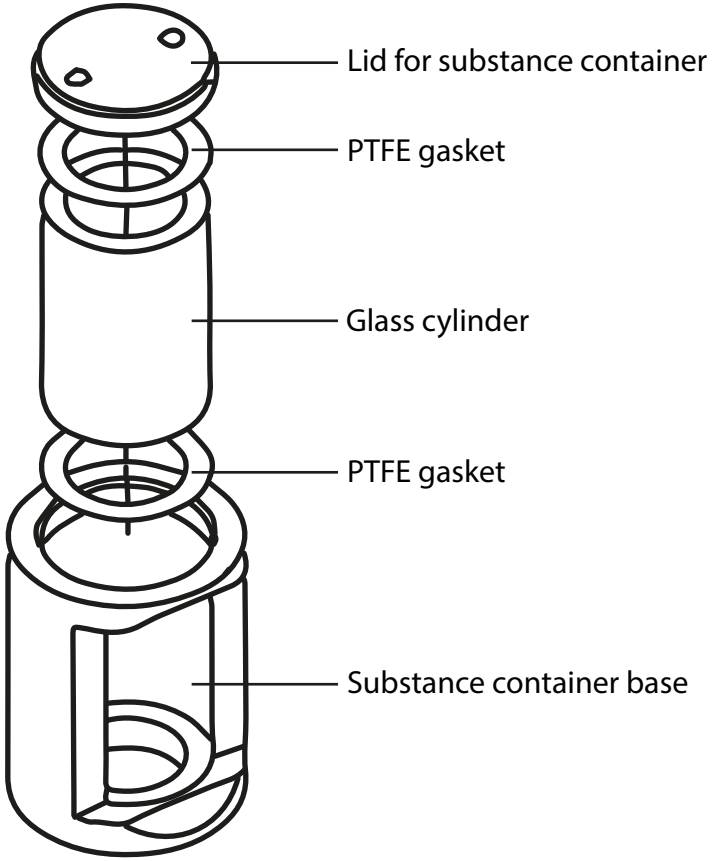

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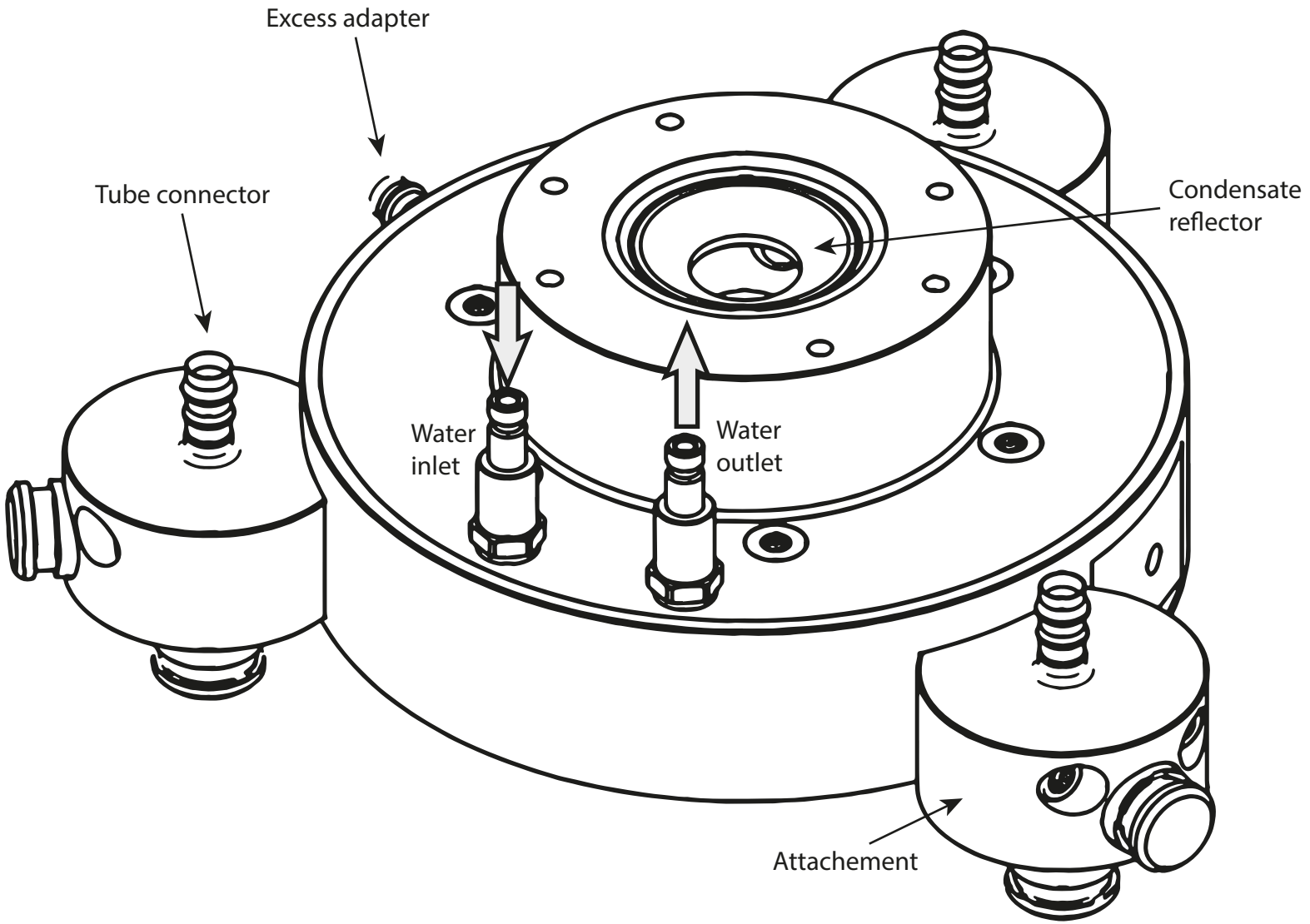
A



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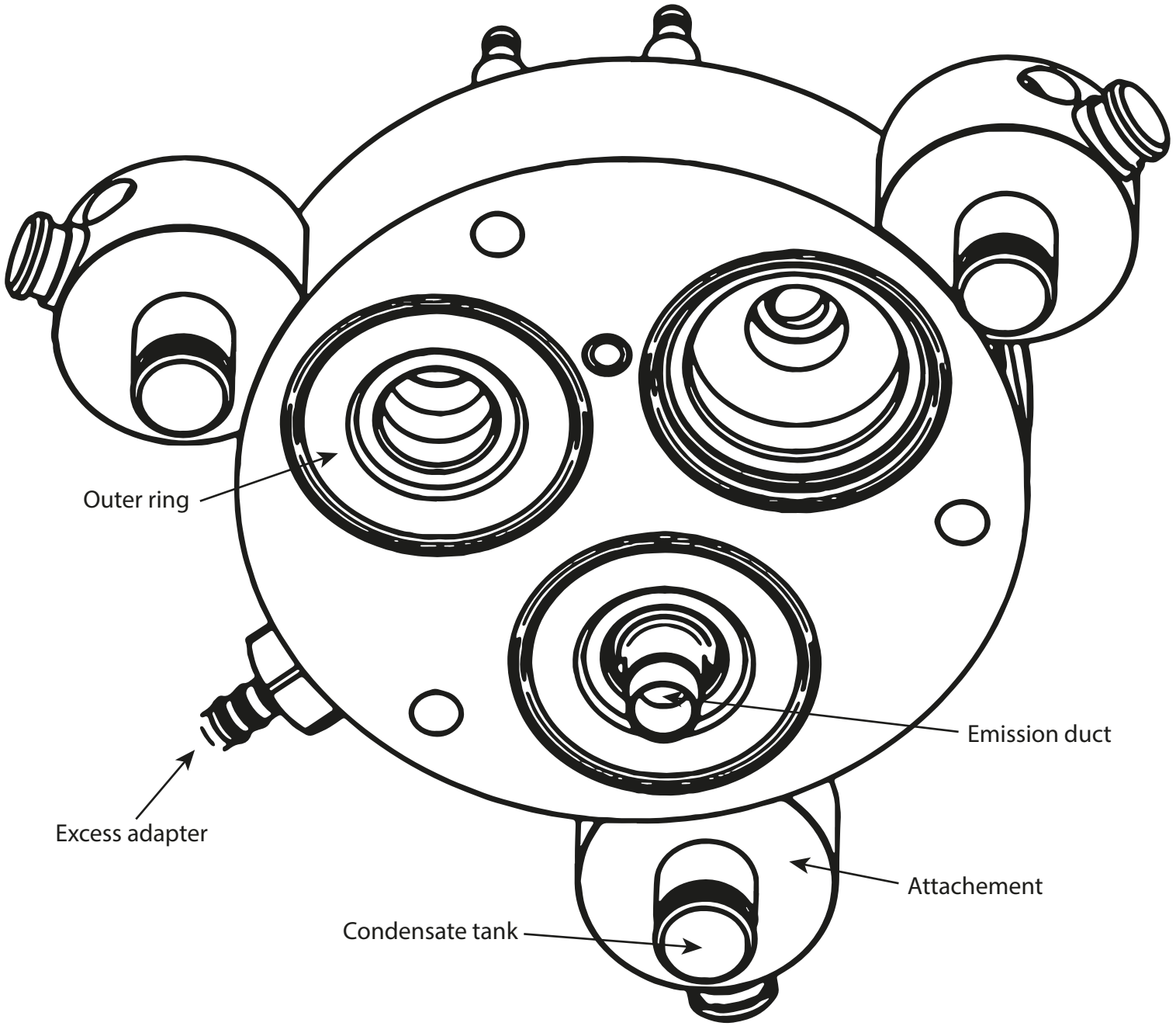
B



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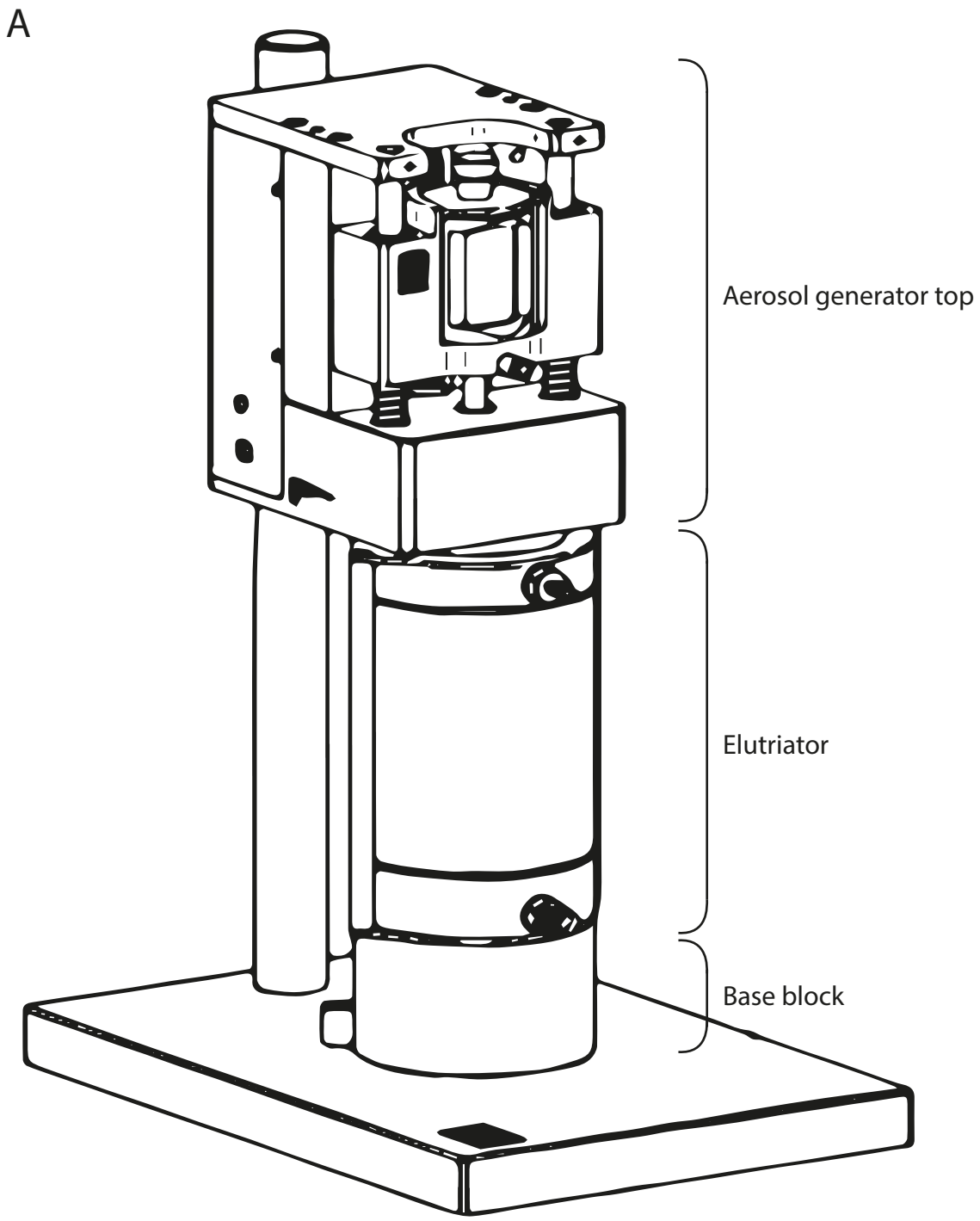

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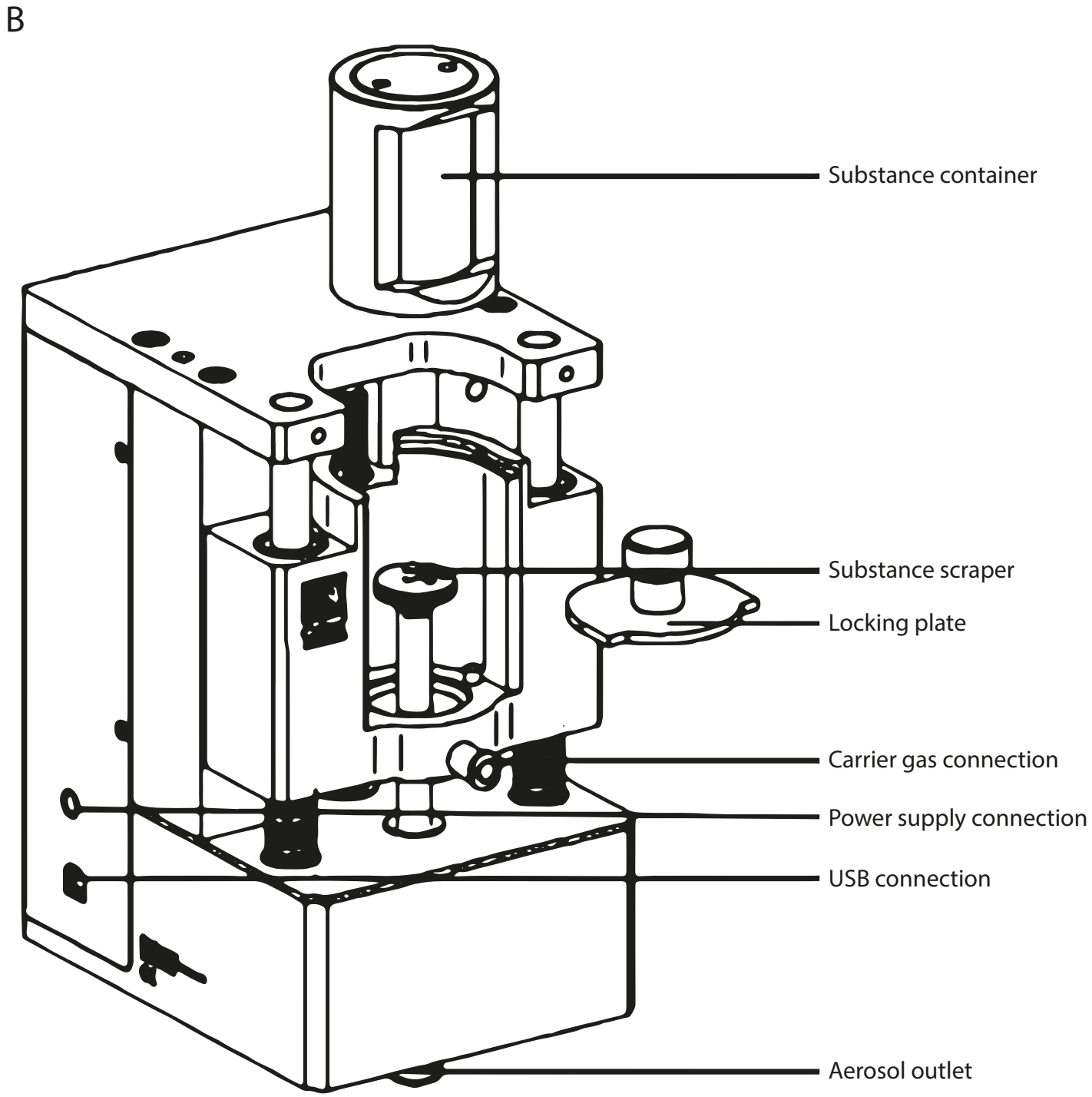

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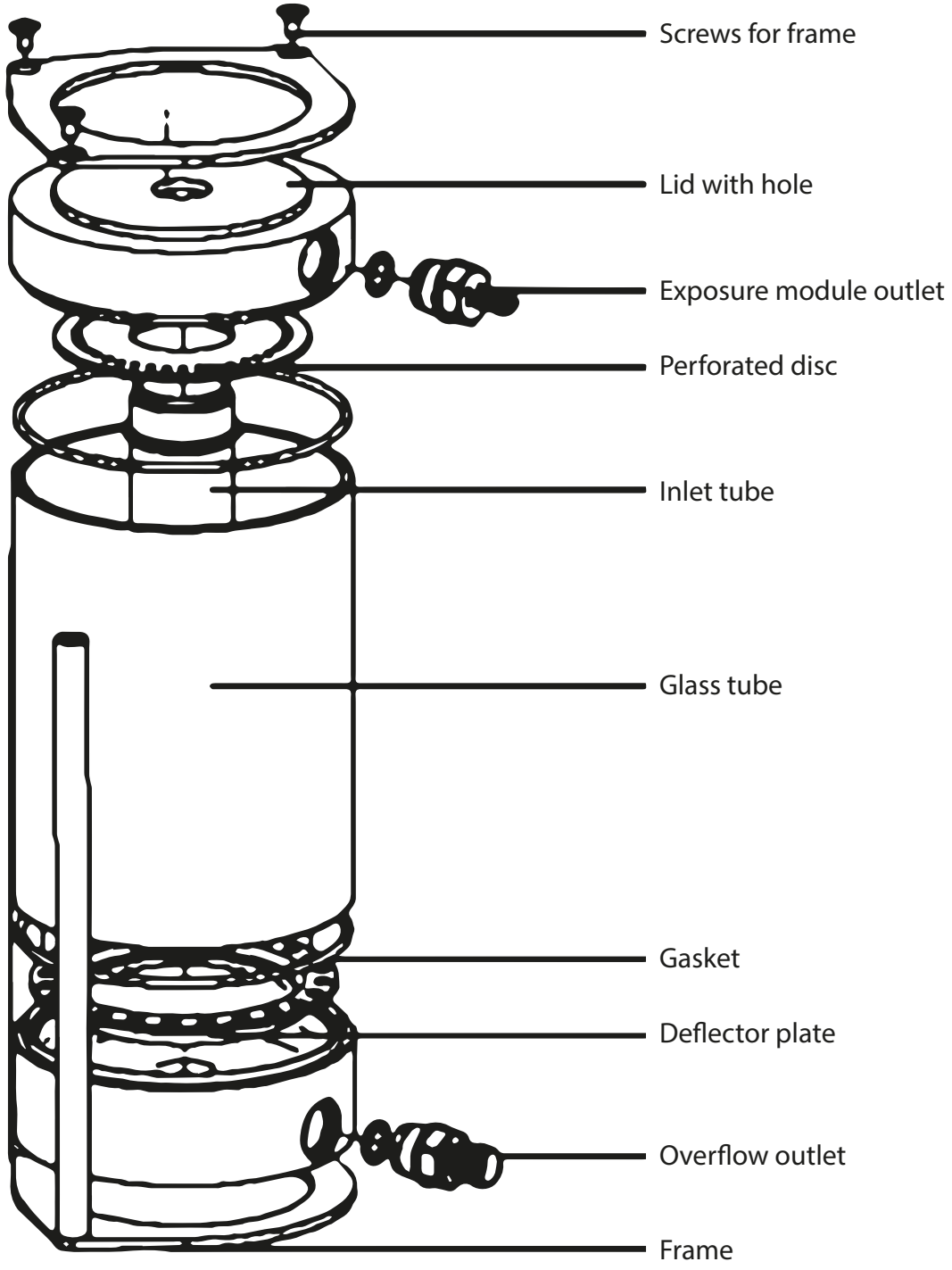
C



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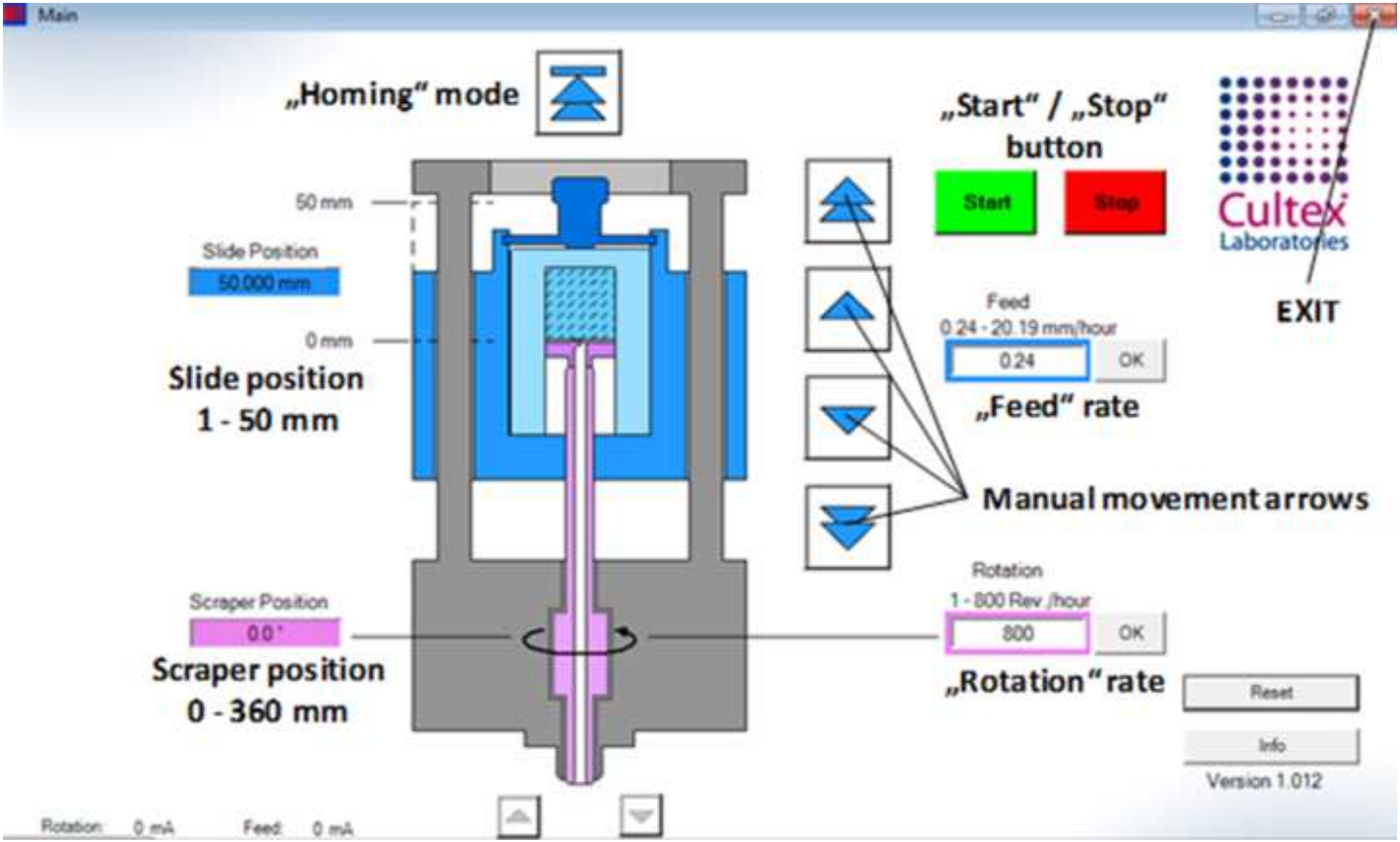

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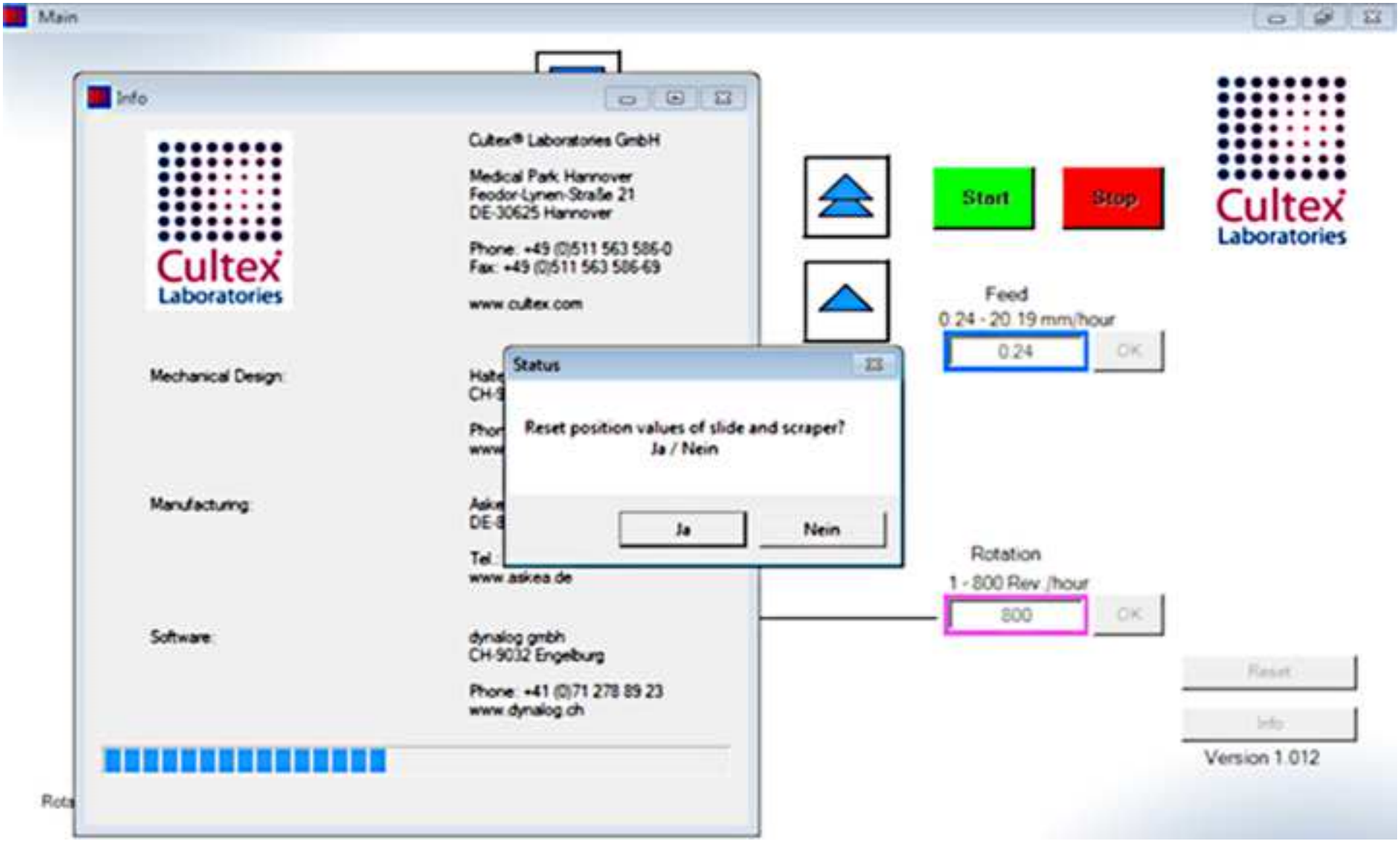

Table

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Table 1: Accordance between *in vivo* and *in vitro* results.

Substance	OECD TG	Reference results *in vivo*		Results *in vitro*		Accordance *in vivo / in vitro*
		CLP regulation	Toxicity	PM 50%	PM 75%	
Tungsten(IV) carbide	403	n. c	0	0	0	Yes
Tungsten(IV) carbide nano	-	n. c.	0	0	0	Yes
N,N'-ethylenebis(N-acetylacetamide)	EPA OPPTS 870.1300	n. c.	0	0	0	Yes
Silicon dioxide	403	n. c.	0	0	0	Yes
Diammonium hydrogenorthophosphate	403	n. c.	0	0	0	Yes
Disodium fluorophosphate	403	n. c.	0	0	0	Yes
Neodymium oxide	436	n. c.	0	0	0	Yes
Potassium hydrogen monopersulfate	403	n. c.	0	0	0	Yes
Cycloheptapentylose	403	n. c.	0	0	0(2x) / 1(1x)	(Yes)
Vanadium(III) oxide	403	n. c.	0	0	0(2x) / 1(1x)	(Yes)
Tetrapotassium pyrophosphate	403	n. c.	0	1	1	No
Tetrabromophthalic anhydride	403 (similar)	n. c.	0	1	1	No
Cetylpyridinium chloride	403	Acute Tox. 2	1	1	1	Yes
N-Lauroylsarcosine sodium salt	403	Acute Tox. 2	1	1	1	Yes
Zinc dimethyldithiocarbamate	403	Acute Tox. 2	1	1	1	Yes
Copper(II) hydroxide	403	Acute Tox. 2	1	1	1	Yes
Zinc selenite	436	Acute Tox. 3	1	1	1	Yes
Sodium metavanadate	403	Acute Tox. 4	1	1	1	Yes
Divanadium pentaoxide	403	Acute Tox. 4	1	1	1	Yes
Cadmium telluride	403	Acute Tox. 4 (n. c.)	1	0	0	No

n. c. = not classified; 0 = non-toxic; 1 = toxic; CLP = classification, labelling and packaging

Table 2: Table of materials.

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Cells			
A549	ATCC	CCL-185	
Cell culture medium and supplies			
DMEM	Biochrom, Berlin, Germany	FG 0415	used as growth medium
DMEM	Gibco-Invitrogen, Darmstadt, Germany	22320	used as exposure medium
FBS superior	Biochrom, Berlin, Germany	S 0615	
Gentamycin (10mg/mL)	Biochrom, Berlin, Germany	A 2710	
HEPES 1M	Th. Geyer, Renningen, Germany	L 0180	
PBS	Biochrom, Berlin, Germany	L 1825	
Trypsin/EDTA (0.05%/0.02%)	Biochrom, Berlin, Germany	L 2143	
Cell culture material			
CASY Cups	Roche Diagnostic GmbH, Mannheim, Germany	REF 05651794	
Cell culture plates	Corning, Wiesbaden, Germany	3516	6-well plates
Corning Transwell cell culture inserts	Corning, Wiesbaden, Germany	3450	24mm inserts; 6-well plates; 0.4 µm
Chemicals			
CASYton	Roche Diagnostic GmbH, Mannheim, Germany	REF 05651808001	
Compressed Air (DIN EN 12021)	Linde Gas Therapeutics GmbH, Oberschleißheim, Germany	2290152	
WST-1	Abcam, Cambridge, United Kingdom	ab155902	
Instruments + equipment			
CASY Cell Counter	Schärfe System GmbH, Reutlingen, Germany		
Circulation thermostat	LAUDA, Lauda-Königshofen, Germany	Ecoline RE 100	
CULTEX HyP - Hydraulic Press	Cultex® Technology GmbH, Hannover, Gemany		
CULTEX insert sleeve	Cultex® Technology GmbH, Hannover, Gemany		
LTEX RFS - Radial Flow System Type 2 (module for particle exposu	Cultex® Technology GmbH, Hannover, Gemany		
TEX RFS - Radial Flow System Type 2 (module for clean air expos	Cultex® Technology GmbH, Hannover, Gemany		
CULTEX supply			
Flow controller 0-30 ml/min (IQ-Flow)	Bronkhorst Deutschland Nord GmbH		
Flow controller 0-1,5 l/min (EL-Flow)	Bronkhorst Deutschland Nord GmbH		
Filters (large)	Munktell & Filtrak GmbH, Sachsen, Germany	LP-050	erile Filter; Particle retention efficiency > 99,999%
Filters (small)	Parker Hannifin Corporation, Mainz, Germany	9933-05-DQ	Balston disposable filter
Medium pump	Cole-Parmer GmbH, Wertheim, Germany	Ismatec IPC High Precision Multichannel Dispenser	digital peristaltic pump
Microplate Reader Infinite M200 Pro	Tecan Deutschland GmbH, Crailsheim, Germany		
Vakuum pump	KNF, Freiburg, Germany	N86 KT.18	
Vögtlin mass flow controller 0,2-10 l/min	TrigasFI GmbH	Vögtlin red-y compact regulator, Typ-Nr.: GCR-C3SA-BA20	
Water Bath	LAUDA, Lauda-Königshofen, Germany	Ecoline Staredition RE 104	

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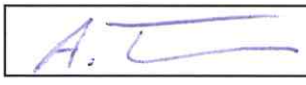
Signature:	
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Editor:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We thank the editor for this advice. We have proofread the manuscript thoroughly in order to avoid any spelling or grammar issues.

2. Please remove Cultex from the title.

Done! We removed the word CULTEX® from the title.

3. Please remove trademark (™) and registered (®) symbols from the Table of Materials.

We removed all trademark (™) and registered (®) symbols from the Table of Materials except for the registered company name "CULTEX® Technology GmbH".

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: CASY, Vogtlin, Woulff, etc.

We thank the editor for this hint. We replaced the commercial language from our manuscript with generic terms. All commercial products, such as CASY or Vögtlin, are now sufficiently referenced in the "Table of Materials".

5. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "Cultex" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language. It is currently used 56 times in the manuscript and must be reduced drastically in order to fit publication standards. 9 of the uses are in the affiliations so please reduce the other 45 uses. Please remove Cultex from the title.

We agree with the editor, that the video narrative should be objective and not as an advertisement for a specific product. We therefore reduced the number of the term "CULTEX" and used it infrequently within the text. It is now used 36 times in the manuscript, but 9 of the uses are in the affiliations, 10 of the uses are in the literature and 9 of the uses are in the disclosure. The term CULTEX® is only written 7 times within the manuscript text and only when directly relevant. Moreover, we removed the term CULTEX® from the title and the keywords.

6. Please remove overly commercial statements such as "very robust", "well predictive", etc. The manuscript cannot become advertisement for the Cultex system.

Done! We removed all overly commercial statements.

7. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Done!

8. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

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We have obtained all copyright permissions for the reuse of the figures from the previous publications. Moreover, we have provided all figures in the adobe illustrator ("ai") format, whenever possible. Fig. 3 (Cultex setup), however, cannot be converted because it is a pixel photo.

10. Please be more neutral in tone in the discussion to avoid the manuscript becoming commercial advertisement for Cultex. Please present limitations of the Cultex system.

We have added aspects concerning limitations and challenges of the Cultex system.

11. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Done! We have changed the references as recommended.

12. Please do not abbreviate journal titles.

Done!

Reviewer #1:

Summary:
**Change exposure "set-up" to exposure "system" (please replace throughout manuscript)*

Done! We have substituted the word “set-up” for the word “system”.

Abstract:
**Lines 3-4 "thus showing…..in vivo" - make clearer e.g. "providing a realistic physiological exposure of the apical surface of the human alveolar region to air".*

Done! We added the term “providing a realistic physiological exposure of the apical surface of the human alveolar region to air" to the sentence.

**Lines 14-15 add "that would normally provide this toxicological assessment" after experiments.*

Done!

Introduction:
**Line 11 - Define 3Rs*

Done! We have specified the term “3R” in the manuscript.

**Para 3 Lines 7-8 - for clarity ensure what is described in the text for Figure 2 is the same as what is labelled on the figure (do for all figures):*

We thank the reviewer for this valuable comment. We checked all text passages describing the figures for consistency with the captions and the labeling on the figures and adapted the manuscript.

**Para 3 Line 10 - unclear what "radial" means here?*

It is correct that the word “radial” is a little bit unclear in this context. We reword the sentence and hope this will contribute to a better understanding.

**Para 4 - This paragraph would provide the reader with a much clearer understanding of the study and its objectives if there was more detail e.g. state which cells were used and why (explain use of A549 cells rather than primary cell lines, including any limitations); state the number of dusty substances studied; how were the exposure doses determined? How long were the exposures? How big were the inserts that were used (to help the reader better understand the exposure doses); provide detail of the in vivo data used to assess concordance; explain why only cell viability was used rather than a combination of outputs such as membrane integrity and cytokines.*

We thank the reviewer for this advice. We added additional information regarding the cell line A549, more details of the used cell culture inserts and the number of test substances and a more precise description of the exposure dose and exposure time. Furthermore, we provided more details about the *in vivo* data and why we used only cell viability as endpoint measurement.
We hope this will help the reader to understand the study much clearer.

**Para 5 - add "the tested" airborne particles, since the authors cannot claim this is the case for untested chemicals*

Done! We have added “the tested” to the sentence.

Protocol:
**2.5 - "determine" the cell number*

Done!

**3.2 - Is "mess" correct?*

Thank you for this hint. Mess is not correct and we replaced “mess” with “measurement”.

**4.1 - what does "under the sterile bench" mean*

We reworded this sentence.

**5 - remove "that is ….frame". The SI unit of force is the N not Kg. The technical data sheet for the Cultex HyP states 5-18 KN, where does the 200-1700 Kg come from?*

We removed “that is … frame”. We also changed the unit of force from Kg to kN. According to the technical data sheet, the CULTEX® Hyp press has a selectable pressure with a range of 5 – 18 kN (equal to 500 – 1800 Kg). The press, however, can also be used for lower pressures up to 200 Kg.
We actually thought that pressing forces of 200 to 1700 Kg would be sufficient to cover all the pressing forces we need for our test substances. Unfortunately, the substance container broke down by pressing forces higher than 1000 Kg. We therefore pressed all test substances in the range of 200 – 1000 Kg.
We have adapted the pressing force and added this limitation to the manuscript.

**5.3 - Check figure and text are consistent eg glass tube or cylinder? Is it the same as the "plunger"?*

We checked figures and text for consistency and replaced the word “glass tube” with “glass cylinder”. The plunger, however, is not the same as the glass cylinder. The plunger, made of either plastic or stainless steel, is used to press the test substance into a powder cake within the glass cylinder, which is part of the substance container.

*5.4 - unclear what "returning it slightly" means

Thank you! We deleted this part of the sentence, because it doesn’t provide a better understanding.

**6.1 - are the mass flow controllers or the Woulff's bottle shown in Figure 3?*

No, but they will be shown during the video narrative.

**6.2 - on the attachments of what - the aerosol guiding module?*

Correct. We mean the attachments of the aerosol guiding module and added this information.

**6.4 - explain role of the Elutriator*

The Elutriator is a reservoir for the generated particulate atmosphere, designed in such a way that particles bigger than 7 µm were deposited within the reservoir, whereas smaller particles were transported to the exposure module to interact with the cells.
We added this description of the Elutriator to the manuscript.

**6.5 - change "disposal" to "removal" (also 8.3)*

Done!

**7.3 - current should be reported as Amps (SI units), is this referring to flow?*

We have replaced the word “current” with “flow”.

**8 - unsure what is meant by "impermeability", is this testing for leaks within the system?*

The impermeability check is used to ensure that the module has been reassembled properly and that there are no leaks within the system. We replaced “impermeability” with “leakage” and hope this contributes to a better understanding.

**10.5 - blind or blank inserts?*

Blind insert.

Results:
**Line 6 - add "of the tested compounds" after hazards*

Done!

**Line 24 - some detail on the in vivo data used as a comparison would be helpful for the reader. Was it acute toxicity data in animal's e.g LCt 50 or related parameters?*

We thank the reviewer for this advice. We added the additional information that all *in vivo* data are based on at least one reliable study according to OECD test guideline (TG) 403 or TG 436. The TG 403 and TG 436 use a traditional LC50 protocol and a concentration x time (C x t) protocol.

**Final sentence - add "the selected" airborne particles*

Done!

Figure Legends:
**Ensure consistency between the figure legends, figures and descriptive text throughout eg Figure 2 legend has "the sampling and locking module" while the figure itself is just labelled as "sampling module". Figure 3 legend mentions the Elutriator but this is not labelled on the figure itself.*

We have checked the whole manuscript for consistency between figure legends, figures and the descriptive text.

**There is no legend for Table 2 - Table of materials*

We have added a legend for table 2.

**Supplementary figure 2 should be labelled as A) and B) in line with the legend (also for suppl fig 3). Is B the aerosol guiding module or the sampling module?*

We chanced the labelling for the figures as recommended. Fig. 2B shows the bottom view of the aerosol guiding module. This is also described in the figure legend.

Figures:
**Ensure consistent labelling with legend and text for all figures*

Done!

**Figure 1 - Inlet of what? Explain blue circles*

We have explained Figure 1 in more detail by describing the inlet and the blue circles.

**Figure 2 - move inlet adapter and aerosol guiding module to left above sampling module*

Done!

**Figure 3 - take out "according to Wright"; is the aerosol reservoir the same as the Elutriator? Label the sample container*

The aerosol reservoir is the Elutriator. We have adapted the figure by labelling the aerosol reservoir as Elutriator.

**Figure 4 - what is the scale (OD)? Could this be changed to cell viability as figure 5? While there is good inter-lab consistency, there is still variability between labs*

OD is the abbreviations for “optical density”. The results of viability measurements using a microplate reader are normally expressed as Optical Density (OD). However, we changed the scale to viability as recommended.
The central message of this figure is, that exposure of cells to clean air resulted in highly stable and reproducible results across the three laboratories without any decrease of cell viability over time.
The fluctuations of the raw data between the labs are not unusual as the data were collected manually. Moreover, the labs were using different microplate reader, leading to different high OD-values. Normalizing the viability data to the respective incubator controls, however, resulted in a very good consistency not only within but also between the labs.
We modified this figure by changing the scale to viability underlining the good inter-lab consistency.

**Table 1 - need to explain what OECD guidelines 403 and 436 refer to; need to give detail of acute tox categories 2,3 and 4 (fatal, toxic, harmful); the table is a little unclear, need better separation of the in vivo from the in vitro sections*

We modified Table 1 by giving more information on the OECD TG 403 and 436 and the acute tox categories 2-4. Moreover, we separated the *in vivo* from the *in vitro* section more clearly.

**Table 2 needs sorting out into a table, with a legend*

We added table 2 (table of materials) and the corresponding legend.

**Suppl fig 1 glass tube or cylinder; fig 2.1 change arrow direction of water outlet B; fig 2.2 is this the sampling module? 3A label substance container*

We modified and labelled the figures as recommended. Suppl. Fig 2.B depicts the bottom view of the aerosol guiding module and not the sampling module. For a better overview, both the aerosol guiding module and the sampling module are already shown in Figure 2. We did not label the substance container in Suppl. Figure 3A, because it is shown in Suppl. Figure 3B in a larger view.

Discussion:

**Para 2 -"better suited" - expand, for what; has the homogeneity of the particle deposition been proven, if so please reference. Would it not depend on the physicochemical properties of each test dust material? Did the authors confirm homogeneity of deposition for their test dusts?*

We thank the reviewer for this valuable advice. We expanded for what ALI inhalation models are better suited compared to the submerged exposure technique.
The homogeneity of the particle deposition was assessed with in-line filters and has been proven. For achieving a homogenous particle deposition onto the inserts, a diffusor nozzle was placed in the CULTEX® RFS modules 10 mm below the top edge of the conducting hose-connecting point. The corresponding reference is added to the manuscript.
A general characterization of the particles (particle number and mass distribution) was done with an Aerodynamic Particle Sizer APS (#3321, TSI Incorporated) by the reference laboratory CULTEX® technology GmbH (formerly CULTEX® laboratories GmbH), showing that the physico-chemical properties of the dust material had no influence on the homogeneity of the particle deposition.
However, the press characteristics of a test substances can be effected by the physical and chemical properties, leading to the use of different press plungers (either made of plastic or stainless-steel), scraping blades or feed rates. The right choice of the press plunger, the knife and the feed rate were determined for every single test substance in preliminary experiments.

**Para 5 - 1000 kg is not the same as the 200-1700 kg previously stated, this is a little confusing. There is confusion over the pressing force or the pressing pressure?*

We thank the reviewer for this advice. As mentioned above, we actually thought that pressing forces from 200 to 1700 Kg would be sufficient to cover all the pressing forces we need for our test substances. Unfortunately, the substance container broke down by using pressing pressures higher than 1000 Kg. We therefore pressed all test substances in the range of 200 – 1000 Kg.

We have adapted the pressing force (which is equal to pressing pressure) and added this limitation to the manuscript.

**Para 7 - perhaps state OECD 403 and 426 here.*

Done!

Reviewer #2:

We thank Reviewer 2 for the kind comment.

Reviewer #3:

We thank Reviewer 3 for the suggestion to accept our manuscript.

Reviewer #4:

Major Concerns:

1. The authors have a preference in using the WST-1 assays for conducting cell viability assessments with A549 cells. In my experience with this assay, this is not an appropriate assays to use the way it is described in this protocol. The WST-1 assay is optimized for the 96-well format using a 1:10 solution and typically working with cells densities of <100k cells/well. Under this 96-well format, the WST-1 assay produces a good linear response curve. However, when working with larger formats and with higher cell counts leads to the WST-1 response curve to not be linear and plateau fairly quickly. This leads to working under the flat response curve (exceeded the upper limits) where it is very difficult to assess changes in viability. In this protocol, the authors have actually increased the WST-1 concentration used to a working solution of 1:7. I would think they should have used a less concentrated solution since they have a lot more cells in their samples. I don't think this protocol is acceptable.

We thank the reviewer for this valuable comment. We invested much effort at the beginning of our validation study in order to find an appropriate viability assay depending on cell number / cell density within the cell culture inserts, dilution rate and incubation time of the viability reagent. We found that viability assays from different suppliers resulted in different high OD values, although performing according to the manufacturers protocols. Based on the results of many preliminary tests, we decided to use a WST assay from a certain company, using a cell density of 300000 cells / cell culture inserts, a dilution rate of 1:7 and an incubation time of 60 min. Several tests have shown that all three labs were still in the linear range of the curve and not in the saturation range. Thus, the assay is appropriate to use as described in our protocol.

2. The authors do not use any positive controls in their protocols, at least not stated here. While clean air exposures are used as a negative control, where is the positive control for their assays or exposures?

It is correct, that we did not explicit stated any positive controls in this manuscript, but we used both negative and positive controls, as described in the publication which we are referring to (Tsoutsoulopoulos et al., 2019).
Clean air is used as negative control during all exposure experiments, as cells are exposed to a test substance and clean air in parallel, using one module for particle exposure and one module for clean air exposure. Exposure of cells with clean air served primarily as control that exposure has no harmful impact on cell viability.
Within the pre-validation study, lactose monohydrate was chosen as negative control, as lactose is routinely used as pharmaceutical vehicle with practically no known acute toxicity in healthy adults. As expected, exposure of cells with lactose did not show any toxic effect in our exposure system.
Copper sulfate was found most toxic in the exposure experiments of the pre-validation study.
At the beginning of the validation study, exposure experiments with chosen substances of the pre-validation study (e.g. lactose monohydrate and copper sulfate) were repeated to examine whether the findings of the pre-validation study could be confirmed.

3. Figure 4 is intended to show reproducibility and stability in clean air exposures. However, I see a lot of variability in Lab 1 and Lab 3 results within exposure time. Results compared between Lab 1 and Lab 3 are also different. Data from Lab 2 looks very good compared to others, which contradicts the statements that clean air exposures are reproducible and of high quality as described in the text.

We thank the reviewer for this advice. This issue was also raised by reviewer 1.
It seems that there is some inter- and intra-laboratory variability within the exposure time but this is only due to scale of the y-axes which indicates the OD raw data. The fluctuations of the raw data within one lab are not unusual as the data were collected manually. The variability of the OD raw data between the labs appears because every lab was using a different microplate reader.
The main message of this figure is, however, that clean air exposure of cells resulted in no decrease of cell viability over time across all three laboratories. Normalizing the viability data to the respective incubator controls resulted in a very good consistency not only within but also between the labs. We modified the figure by changing the scale to viability underlining our statement that clean air exposures are reproducible and of high quality.

4. Figure 5 is intended to show the dose response of 3 different test substances of differing toxicities (no toxicity, medium toxicity, and strong toxicity). The authors claim that this Cultex RFS system can replace the animal model, however this figure shows a poor representation of an in vitro system replacing the animal model. For each test substance there are 3 doses. In all cases, the dose response curve is just a flat line. The response is the same regardless of dose. If the in vitro system is supposed to replace an animal model, then supporting data for the same substance tested should show responses from doses that produce no adverse effects, some adverse effects, and high adverse effects. What this figure tells me is that dose does not matter. Is there other figures that would better demonstrate the dose response?

We thank the reviewer for this valuable hint. We agree that the selection of the test substances is not well suited for demonstrating the dose response. We exchanged the figures and are presenting now a high toxic and a none-toxic substance, and a substance with medium toxicity showing a good dose response curve.

5. The doses used in Figure 5 are extremely high for ALI exposures. The authors never explain how dose is determined. The authors never explain how long the exposures are conducted for given the target dose. I'm making the assumption that a dose of 25 ug/cm2 was delivered in 15 mins, a dose of 50 ug/cm2 was delivered in 30 mins, and a dose of 100 ug/cm2 was delivered in 60 mins. Is this correct? As stated before, doses are very high. This means that the particle generation system is generating very large concentrations of particles (likely in the tens of mg/m3 in air). What are the particle characteristics? With such high particle concentrations, they are likely agglomerating resulting in very large aerodynamic particle sizes. What happens if users want to use low particle concentrations? Would they have to test for much longer exposure times?

The reviewer 2 is correct with his assumptions that the exposure dose is correlated to the deposition time.
We exposed the A549 cells to 20 different test substances with 25 µg/cm²/15min over three various deposition periods resulting in three different exposure doses of 25 µg/cm², 50 µg/cm² and 100 µg/cm² after 15, 30 or 60 min, respectively.
The deposited particles, however, were not washed off after deposition, but remained on the cells for 24 h. The deposition times of the particles were therefore 15, 30 and 60 min, but the exposure of the cells lasted for 24 h in total. We have added these additional information regarding the exposure duration and dosage to our manuscript.
We decided to use these dosages in dependence on the pre-validation study and the NanoCare study (Kroll A et al., 2011). To achieve the required levels of deposition, a characterization of every substance with regard to the physical deposition properties was conducted in the reference laboratory. Particle and press characteristics depend thereby on the physical and

chemical properties of a test substance such as strength, stickiness, or the fineness and coarseness of the particles.
If particles would agglomerate and get bigger than 7 µm, they would not reach the cells. The particles are generated by scraping a thin layer from a pre-formed powder cake using the aerosol generator. The generated particle mass are then guided into the Eluatriator by a constant airflow, which serves as a reservoir for the generated particulate atmosphere, designed in such a way that particles bigger than 7 µm are deposited within the reservoir. Smaller particles are transported to the exposure module to interact with the cells. We modified the manuscript by adding this explanation of the role of the Elutriator.
Adjusting the deposited particle mass (= dose) can be done by either increasing the speed of scraping or by extending the time of particles deposition at a constant speed rate of scraping. Based on our experience, we know that faster scraping affects particle size, thus having significant impact on particle mass distribution. Thus, we have chosen a constant scraping rate and three different exposure times resulting in a deposition of three different exposure doses.
If users want to use low particle concentrations, they can either reduce the speed of scraping or the time of particle deposition.

6. Since authors state that exposures of 60 mins are considered prolong exposures, does this mean this exposure system cannot be used for exposures longer than 60 mins? After 60 minutes, a lot more issues of cell desiccation will become evident. How to avoid this?

No, the exposure system can be used for cell exposures (more precisely the time of particle deposition) longer than 60 min. Within the validation study, deposition of particles onto the cells was done for 15, 30 and 60 min as we wanted to keep the same exposure durations and exposure doses used in the pre-validation study.
The CULTEX® RFS, however, is only an *in vitro* model system without any clearance mechanisms. Exposure of cells inside the exposure module longer than 60 min are feasible, but special focus has then to be set on the deposited particle mass. Cells can die after particle deposition due to a lack of oxygen if they are covered by a too high particulate mass, although the substance itself may not be toxic.
Cell desiccation can become evident but is avoidable by using appropriate cell culture inserts. Cell culture inserts can have a crucial influence on permeability and humidity. A former study showed that cell culture inserts with three side wall openings resulted in an increase in relative humidity inside the cell culture insert when used in the CULTEX® RFS (Tsoutsoulopoulos et al., 2016). In addition, these inserts provided a better media supply due to a 2-fold higher number of pores compared to cell culture inserts from another company.

Minor Concerns:

1. A549 cells are cultured at a density of 300,000 cells/well and allowed to grow for 24h before exposure in a 6-well Transwell. Given doubling time of 20-24 h in this cell type, this means at the time of exposure there are ~600,000 cells. This is a bit on the lower end where cells would likely be at ~85% confluency. In ALI exposures, having a confluent cell monolayer is desired. Letting cells grow for 48h or 72h before exposures would be better to allow the cells to undergo 2 or 3 cell doublings within the insert.

We thank the reviewer for this calculation, which is completely right. It is correct, that the cells had a confluency of about 85 % at the time of exposure. However, we decided not to use a higher cell density than 3x10^5 cells/well, a longer cultivation time or a cell confluency of 100 %

at the time of exposure. Using our exposure protocol, cells were seeded at day 1, cultivated for 24 h and exposed at day 2. Cell viability was then measured 24 after exposure at day 3. Seeding more cells than 3x10^5 would have led to a too high cell density at the time of viability measurements for the clean air exposed cells and the incubator controls (cells that were not exposed to particles or clean air). We therefore decided to culture cells with the parameters given above.

Moreover, our results showed that exposing cells with a confluency of about 85 % to clean air did not affect the viability of the cells.

References

- Kroll A et al., Cytotoxicity screening of 23 engineered nanomaterials using a test matrix of ten cell lines and three different assays. Part Fibre Toxicol. 8 (2011)
- Tsoutsoulopoulos et al., Optimization of the CULTEX® radial flow system for in vitro investigation of lung damaging agents. Toxicol Lett. 244:28-34 (2016)
- Tsoutsoulopoulos et al., Validation of the CULTEX® Radial Flow System for the assessment of the acute inhalation toxicity of airborne particles. Toxicol In Vitro. 2019 Aug;58:245-255.

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The CULTEX RFS: A Comprehensive Technical Approach for the *In Vitro* Exposure of Airway Epithelial Cells to the Particulate Matter at the Air-Liquid Interface

Michaela Aufderheide,[1,*] Beat Halter,[2] Niklas Möhle,[1] and Dieter Hochrainer[3]

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Abstract

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The EU Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) demands the implementation of alternative methods for analyzing the hazardous effects of chemicals including particulate formulations. In the field of inhalation toxicology, a variety of *in vitro* models have been developed for such studies. To simulate the *in vivo* situation, an adequate exposure device is necessary for the direct exposure of cultivated lung cells at the air-liquid interface (ALI). The CULTEX RFS fulfills these requirements and has been optimized for the exposure of cells to atomized suspensions, gases, and volatile compounds as well as micro- and nanosized particles. This study provides information on the construction and functional aspects of the exposure device. By using the Computational Fluid Dynamics (CFD) analysis, the technical design was optimized to realize a stable, reproducible, and homogeneous deposition of particles. The efficiency of the exposure procedure is demonstrated by exposing A549 cells dose dependently to lactose monohydrate, copper(II) sulfate, copper(II) oxide, and micro- and nanoparticles. All copper compounds induced cytotoxic effects, most pronounced for soluble copper(II) sulfate. Micro- and nanosized copper(II) oxide also showed a dose-dependent decrease in the cell viability,

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