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A New Cell Culture Exposure System for Studying the Toxicity of Volatile Chemicals at the Air-Liquid Interface

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Abstract

A cell culture exposure system (CCES) was developed to expose cells established at an air-liquid interface (ALI) to volatile chemicals. We characterized the CCES by exposing indigo dye-impregnated filter inserts inside culture wells to 125 ppb ozone (O₃) for 1 h at flow rates of 5 and 25 mL/min/well; the reaction of O₃ with an indigo dye produces a fluorescent product. A 5-fold increase in fluorescence at 25 mL/min/well versus 5 mL/min/well was observed, suggesting higher flows were more effective. We then exposed primary human bronchial lung epithelial cells (HBECs) to 0.3 ppm acrolein for 2 h at 3, 5, and 25 mL/min/well and compared our results against well-established *in vitro* exposure chambers at the U.S. EPA's Human Studies Facility (HSF Chambers). We measured transcript changes of heme oxygenase-1 (*HMOX1*) and interleukin-8 (*IL8*), as well as lactate dehydrogenase (LDH) release, at 0, 1, and 24 h post-exposure. Comparing responses from HSF Chambers to the CCES, differences were only observed at 1 h post-exposure for *HMOX1*. Here, the HSF Chamber produced a ~6-fold increase while the CCES at 3 and 5 mL/min/well produced a ~1.7-fold increase. Operating the CCES at 25 mL/min/well produced a ~4.5-fold increase; slightly lower than the HSF Chamber. Our biological results, supported by our comparison against the HSF Chambers, agree with our fluorescence results, suggesting that higher flows through the CCES are more effective at delivering volatile chemicals to cells. This new CCES will be deployed to screen the toxicity of volatile chemicals in EPA's chemical inventories.

Keywords

Air-Liquid Interface; Acrolein; Volatile Organic Compounds; In Vitro Exposure; Toxicity Screening; Air Pollution

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Disclosure of Interest

The authors report no conflict of interest.

Introduction

A wide range of *in vitro* and *in vivo* exposure assessment tools have been employed to experimentally assess the toxicological activity of aerosols, vapors, and gases. *In vivo* inhalation exposure studies permit cause and effect assessments to determine the dose range required to induce adverse biological responses. The toxicological studies of air pollutants conducted in a laboratory usually necessitate use of elevated exposure concentrations, relative to what is found in the ambient air. *In vitro* tools offer an attractive alternative, but assessments of airborne contaminants are challenging because cultured cells are often grown in a liquid suspension or adhered to a solid substrate covered with liquid medium. Although cell-based assays can be much more sensitive than *in vivo* assays, conventional *in vitro* dosing methods and systems are not adequate to expose cultured cells in a manner that mimics a more realistic cell exposure to real-world complex mixture (gases, vapors, and aerosols). Traditional *in vitro* dosing methods require, for example, the addition of particulate matter (PM), PM extracts, or chemicals in dimethyl sulfoxide (DMSO) or water into cell culture medium. However, about 10% of chemicals nominated for study in the U.S Environmental Protection Agency's (EPA) Toxic Substances Control Act (TSCA) chemical substance inventory are insoluble in DMSO, water, or are volatile; thus their toxicity cannot be adequately tested using traditional *in vitro* dosing methods. Therefore, EPA has been tasked to develop new methods to screen chemicals in the TSCA inventory, including chemicals such as acrolein, formaldehyde, 1,3-butadiene, and benzene, among many others.

To circumvent the difficulties with exposing cells in culture directly to chemicals or complex mixtures, modified culture techniques establishing cells at an air-liquid interface (ALI) have been used successfully (Doyle et al. 2004; Ebersviller et al. 2012; Lichtveld et al. 2012; Baldridge et al. 2015). The ALI method requires the culturing of cells on a porous membrane where cell culture medium is added to the basolateral side to provide the necessary nutrients for viability, while the apical surface of the cells is exposed directly to the air (Dvorak et al. 2011; Paur et al. 2011; Zavala et al. 2014). In addition to a direct pollutant-to-cell interaction, exposure of cells at ALI preserves the test substance in its natural state, thus mimicking a more realistic exposure scenario.

A standard incubator exposure chamber system housed at EPA's Human Studies Facility (HSF) located in Chapel Hill, NC (henceforth referred to as HSF Chambers) permit the exposure of cells at the ALI. These HSF Chambers have been widely used since 1991 (Becker et al. 1991; Noah et al. 1991; Mckinnon et al. 1993). However, their large foot-print and intricate setup makes it unable to transport them to another laboratory space. We therefore sought to miniaturize our ALI cell culture exposure system so that it can be deployed in various settings at EPA's facilities to screen for various volatile chemicals. Some small-sized ALI exposure systems exist commercially; however they do not meet our specific needs. Typically, these ALI exposure systems have a nozzle to deliver air flow perpendicular to the cells and their operating flow rates are 2–10 mL/min/well (Zavala et al. 2017). These low flow rates are required because using higher flow rates can lead to cytotoxic effects due to cell desiccation. Cell desiccation occurs because these ALI systems do not condition and regulate the sampled air flow to incubator-like conditions, i.e., 37°C and >70% RH. We have previously demonstrated that appropriately conditioning the

sampled air prevents cytotoxicity from occurring (Zavala *et al.*, 2017). We therefore postulate that higher air flows can be used in a similar system and that higher flow rates play significant role in delivering the airborne contaminant to the cultured cells more efficiently.

The purpose of the present study was to miniaturized our existing exposure system (the HSF Chambers) with which to screen the toxicity of volatile organic compounds (VOCs) directly. Here, we demonstrate the utility of a new *in vitro* exposure system that has been optimized for testing of volatile chemicals. This system, which we refer to as the Cell Culture Exposure System (CCES), permits the exposure of cultured cells (lung, nasal, skin, macrophages, etc.) at the ALI condition. We selected acrolein, a TSCA chemical, to demonstrate the utility of the CCES by exposing human lung cells at EPA's Inhalation Toxicology Facility (ITF) located in Research Triangle Park, NC. Furthermore, we compared our results to those obtained from similar exposures in the HSF Chambers.

Materials and Methods

HSF Chambers

A dedicated *in vitro* exposure laboratory at EPA's HSF is equipped with 8 total incubator exposure chambers; 4 are used for filtered air exposures and 4 for test gas exposures. Each incubator exposure chamber is composed of retrofitted incubators that contain an isolated 1 cubic foot stainless steel enclosure containing two perforated shelves. Teflon sample lines connect to a port on the top of the exposure chambers and deliver filtered air or a test gas. Each chamber also has an outlet port to exhaust the exposure air at its bottom. Up to 8 tissue culture plates of any format (i.e., 6-, 12-, and 24-well) can be exposed simultaneously within each chamber. These chambers are operated at a flow rate of 20 L/min under negative pressure. As the air enters the chamber, the velocity is decreased and the air is randomly dispersed as it flows through the perforated shelves. This configuration results in each cell culture insert being exposed to a much slower, turbulent flow with an approximate air exchange rate of 42 per hour. Temperature and relative humidity (RH) are regulated at 37°C and 85%, respectively. These chambers are limited to exposing culture cells to gaseous pollutants (i.e., VOCs, nitric oxide, nitrogen dioxide, carbon monoxide, ozone and sulfur dioxide), and are limited to a fixed location due to the size of the apparatus, which occupies most of the lab space.

Miniaturized Cell Culture Exposure System

The CCES was developed at EPA's Inhalation Toxicology Facility and is comprised of a base module that accommodates a 24-well tissue culture plate with 6.5-mm diameter Transwell inserts (Figure 1). The cover of the base module contains two parallel gas distribution manifolds with inlet nozzles positioned 2 mm above each of the Transwell inserts when placed over the base module. The base module, cover, distribution manifolds, and inlet nozzles were machined out of stainless steel. Using a vacuum pump and precision valves, various flow rates of 72, 120 or 600 mL/min were sampled through this system. Assuming a uniform air distribution through each nozzle, the cells in each well insert are exposed to air, or test gas, at a flow rate of 3, 5, or 25 mL/min/well. Similar exposure systems with air delivered perpendicular to the cells use flow rates <10 mL/min/well (Zavala

et al. 2017). For a 24-well format flow rates <5 mL/min/well are typically used (Zavala et al. 2017). We therefore selected two low flow rates and one higher flow rate to test.

We have previously demonstrated that ALI exposure systems need to appropriately condition the sampled air to 37°C with RH levels above 70% to prevent cytotoxicity from occurring due to the operation of the system itself (Zavala *et al.*, 2017). Therefore, we use a similar approach for the CCES system (Figure 2). Two CCES units, one for control air and one for the test gas, are maintained in a temperature- and humidity-controlled aluminum enclosure that provides the cells an incubator-like environment. Plexiglas doors provide access to the interior of the enclosure where a heater and a fan effectively regulate temperature. Custom-designed in-line diffusion humidifiers upstream of each exposure module permit RH levels to be increased. Thermo-Hygrometers (Model RH411, OMEGA Engineering, Inc., Norwalk, CT) are used to monitor temperature and RH during operation. The entire system is mounted on a cart for ease of transport between laboratories.

Flow Rate Optimization of the CCES

We postulated that the flow rate in these types of ALI exposure systems plays a significant role in delivering the airborne contaminant to the cultured cells, with higher flow rates delivering contaminants more efficiently. We modified a previously-described fluorescence method to detect ozone (O₃) in ambient air (Felix et al. 2011) to permit quantitative measurements of gas delivery to the bottom surface of each well inside the CCES. After extensive characterization and effectiveness testing with the fluorescence-based method to evaluate the CCES, design changes and optimization of operating parameters were applied. We impregnated a 14.3-mm diameter Whatman 41 filter with an indigotrisulfonate (ITS) solution (i.e., a dye). The ITS solutions were made fresh by gently mixing 3.1 mg of potassium ITS (Sigma Aldrich) with 2.5 mL of deionized water and 2.5 mL of ethylene glycol in a 15-mL conical tube. Solutions were used within 4 h of preparation.

Twelve 14.3-mm diameter filters cut from 150-mm diameter Quantitative Filter Papers (Whatman, Cat # 1441–150) were placed directly inside the wells of 24-well plates; filters were evenly distributed over entire plate. Aluminum spacers were added under each filter to elevate its height so that the distance from the filter to the nozzle was 2 mm; the same distance from the Transwell membrane to the nozzles. Once in place, 20 µL of the ITS solution was added to the center of each filter and allowed to spread throughout the filter by capillary action as described previously (Felix *et al.*, 2011). The ITS-impregnated filters were placed inside the CCES, and the filters were exposed to 0.125 ppm O₃ for 1 h at a flow rate of either 5 or 25 mL/min/well. O₃ was generated from an API 401 Model O₃ calibrator, which is a primary standard and is certified annually. Prior to each test, the sample lines and CCES were conditioned with 0.8 ppm O₃ for 1 h to minimize O₃ losses to wall surfaces. A total of 3 independent tests (12 technical replicates per test) were conducted at each flow rate. As O₃ is delivered to each well, ozonolysis of ITS results in fluorescent product that can be quantified (Felix *et al.*, 2011). After exposing impregnated filters to O₃, filters were quickly extracted in 3 mL of MilliQ water inside a glass scintillation tube. Only 12 filters were used per test to minimize the extraction time where filters could be exposed to room air and potential background ozone. A separate set of 3 independent tests were also conducted

using the HSF Chambers under similar conditions for comparative purposes. Tubes were protected from the light, vortexed for 30 sec, and 200- μ L aliquots, in triplicate, were added to a 96-well solid black microplate for analysis. Fluorescence was assessed using the SpectraMax i3 Multi-Mode Microplate Reader (Molecular Devices, LLC).

Preliminary tests for proof-of-concept purposes were conducted to determine the optimal excitation and emission wavelengths by exposing ITS-impregnated filters inside a 25-mL glass midjet impinger at a flow rate of 0.5 L/min for 10, 30, and 60 min to simulate the cell exposure well. The SpectraMax i3 optimization wizard tool spans a wide range of excitation and emission wavelengths to determine the optimal parameters.

Primary Human Bronchial Epithelial Cells

Primary human bronchial epithelial cells (HBECs) were obtained via bronchial brushing from healthy, non-smoking male donors aged 21–40 using a previously published method (McCullough et al. 2014). Donors gave their informed consent after being informed of procedures and associated risks. The consent and collection protocol were approved by the University of North Carolina School of Medicine Committee on the Protection of the Rights of Human Subjects and by the US Environmental Protection Agency. After collection, HBECs were cultured in Bronchial Epithelial Growth Medium (BEGM Bullet Kit, Lonza, Walkersville, MD) on plastic tissue culture flasks and expanded until passage 3. For all exposures, cells were plated on uncoated 6.5-mm diameter (24-well format) Transwell inserts (Corning Life Sciences, Tewksbury, MA) at a density of 0.5×10^5 cells/insert with 0.5 mL of medium on the basolateral side and 0.2 mL on the apical side. Cells were expanded and grown for ~4 days until they reached confluency. The cells were then placed at ALI condition using a 1:1 DMEM-H (Gibco, 11995) and BEGM Bullet Kit medium containing 1.5 μ g/mL BSA and an additional 25 mg of bovine pituitary extract for an additional 4 days as described previously, which allowed them to become polarized and only partially differentiated (Ross et al. 2007). All tissue culture procedures took place at EPA's HSF. For exposures conducted using the CCES, cells were transported on Transwell inserts to the ITF located ~15 miles away, one day prior to exposure. Cells were allowed to recover overnight prior to exposure the following day.

Acrolein Exposures

Two CCES units were maintained inside a heated enclosure (37°C, Figure 2). One unit was used for acrolein exposures, and the second unit was used for sham (air control) exposures. We exposed cells at a flow rate of 3, 5, and 25 mL/min/well in the CCES. This translates to an air exchange rate per well of 53, 88, and 442 per hour, respectively. Similarly, exposures in the HSF Chambers were conducted in two chambers at the fixed 20 L/min flow rate; one chamber for acrolein and one chamber for sham (air control). Certified cylinders of acrolein at a concentration of 1,000 parts per million (ppm) were used as our generation source. We diluted acrolein to 0.3 ppm using medical grade air (the same air used for sham exposures), and mixing flow was monitored using mass flow controllers. A concentration of 0.3 ppm was selected to minimize cytotoxicity to assess subtle gene-expression changes; it was previously shown in A549 cells exposed at ALI that a concentration of ~0.6 ppm did not induce cytotoxicity (Lin et al. 2014). With the CCES at the RTP facility, acrolein

concentration was determined using a gas chromatograph (HP 5890A) with flame ionization detector (FID) and an AT 624 packing column (30-m $\times$ 0.53-mm). With the HSF chambers, acrolein concentration was determined using the Thermo Scientific™ Model 51 i Total Hydrocarbon Analyzer that utilizes FID technology. Preliminary tests analyzing the pH indicated that with the CCES a much lower CO₂ concentration was needed since the air flow is directed straight into cell inserts, whereas in the HSF chambers the air exchange rates are lower. Therefore, we delivered ~2% and ~5% CO₂ with the 0.3 ppm air flow to the CCES and HSF Chambers, respectively, to maintain the proper pH of the culture medium. Since two different analytical methods were employed at two different locations and to ensure the accuracy of the measured concentrations in the presence of CO₂ and high RH levels, an independent audit by the Zedek Corporation confirmed that both analytical methods agreed with each other.

Two hours prior to exposure, the apical surface of the cells was washed with Dulbecco's phosphate buffered saline (DPBS, Life Technologies, Grand Island, NY), and fresh basolateral medium was added. We selected a volume of 0.5 mL in each well because this was enough to surround the Transwell membrane on the outside wall, while minimizing the hydrostatic pressure pushing up on the bottom surface of the membrane. After a 2-h exposure, cells were incubated for an additional 0, 1, or 24 h. We conducted 3 replicate runs with 3 Transwell inserts per chamber for both acrolein-exposed and air-exposed control cells at each time point and at each flow rate.

Cytotoxicity

The amount of lactate dehydrogenase (LDH) released into the basolateral medium was measured at 0, 1, or 24 h post-exposure by subjecting 50- μ L samples of the basolateral medium to the CytoTox Non-Radioactive Cytotoxicity Assay (Promega). We normalized values to those from lysed cells from an unexposed insert, which represented a measure of total (100%) LDH content, to calculate percent LDH release. Fresh culture medium was used to determine a baseline (0%) measurement.

RT-PCR

Interleukin-8 (*IL-8*) and Heme Oxygenase-1 (*HMOX1*) are common sentinel genes for pro-inflammatory and oxidative stress, respectively. We extracted and purified total cellular RNA from three individual Transwell inserts per biological replicate using a Purelink RNA Kit, (Life Technologies). RNA was quantified using a Nanodrop ND-1000 (A₂₆₀/A₂₈₀ 2.0 was considered acceptable). Complementary DNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad) with 1000 ng of purified total RNA per the manufacturer's protocol. We then quantified transcript abundance by TaqMan qPCR with cycling conditions of 95 °C (3:00), 95 °C (0:15), 60 °C (0:45) x40 using a CFX96 Touch real time PCR apparatus (Bio-Rad) with iTaq Universal Probes 2X master mix (Bio-Rad). Fold change of *IL-8* and *HMOX1* transcripts between control air and exposure treatments were calculated, and we normalized values to the abundance of β -actin (*ACTB*) according to the Pfaffl method (Pfaffl 2001). All reactions were conducted in duplex (*IL-8*/*ACTB* or *HMOX1*/*ACTB*) so that *IL-8* and *HMOX1* values were normalized to *ACTB* levels in the same qPCR well. We

ran standard curves for each reaction mixture on each qPCR plate to accurately account for reaction efficiency. Primer and probe sequences are listed in Table 1.

Statistical Analysis

We present data as the mean $\pm$ standard deviation (SD). Fluorescence data was analyzed using a one-way ANOVA with Tukey's post test. Biological data was analyzed using a two-way ANOVA with a Bonferroni post test. The threshold for statistical significance was set at 0.05.

Results

CCES Flow Rate Testing

The following preliminary results demonstrate proof-of-concept for utility of the CCES. Based on the optimization wizard tool of the SpectraMax i3, we determined that an optimal excitation wavelength was 260 nm, and an optimal emission wavelength was 410 nm. ITS-impregnated filters were initially exposed to 0.125 ppm of O₃ using glass midjet impingers for 0, 10, 30, and 60 min at a flow rate of 0.5 L/min. The resulting fluorescence profile was observed at the optimized excitation wavelength (260 nm) and an emission wavelength scanning from 350–500 nm with 10-nm increments (Figure 3).

Once the appropriate parameters for this fluorescence method were finalized, we proceeded to conduct O₃ testing in the CCES with ITS-impregnated filters. For the purpose of comparing a low flow rate to a high flow rate, we opted to use only a flow rate of 5 mL/min/well for the first set of tests. Under this flow rate, a fluorescence measurement of $0.93 \times 10^6 \pm 0.37 \times 10^6$ (mean $\pm$ SD, arbitrary units) was observed (Figure 4); yielding a coefficient of variation (CV) of 39.5%. We postulated that a higher flow rate would deliver the test gas in a more efficient manner; therefore, we selected a flow rate of 25 mL/min/well, a 5-fold increase, for the subsequent test. At this higher flow rate, a fluorescence measurement of $4.8 \times 10^6 \pm 0.99 \times 10^6$ (mean $\pm$ SD, arbitrary units) was observed (Figure 4), which is a 5-fold increase and a reduced CV of 20.5%. This indicated that a test gas can possibly be delivered to the target (i.e., the Transwell insert surface) more effectively and with less variability by increasing the flow rate. In the HSF Chambers, we observed a fluorescence measurement of $1.1 \times 10^7 \pm 1.5 \times 10^6$ (mean $\pm$ SD, arbitrary units) and a CV of 13.5% (Figure 4).

Acrolein Exposures

HBECs were exposed to 0.3 ppm of acrolein using the CCES at EPA's ITF and similarly with HSF Chambers for 2 h. We assessed *HMOX1* mRNA levels at 0, 1, and 24 h post-exposure for all exposure conditions as a marker of oxidative stress. This marker has been routinely used in the HSF Chambers when conducting ALI exposures. The *HMOX1* fold changes obtained from the CCES at various flow rates were compared to the fold change obtained from the HSF Chamber exposures. As shown in Figure 5, there was a significant difference in responses only at the 1 h time point. As can be seen, a ~6-fold increase in *HMOX1* is observed in the HSF Chamber. When the HSF Chamber is compared to the CCES at 3 and 5 mL/min/well flow rates, a ~1.7-change is observed; this is a significant reduction ($p < 0.001$) in response. However, once the flow rate to the CCES is increased to

25 mL/min/well, a ~4.5-fold change is obtained, and when compared to the HSF Chamber, a statistically significant difference ($p < 0.05$) is still observed.

Similarly, we assessed *IL-8* mRNA levels at 0, 1, and 24 h post-exposure for all exposure conditions since this pro-inflammatory marker is a common endpoint for similar ALI exposure studies in the HSF Chambers. The *IL-8* fold changes obtained from the CCES at various flow rates were compared to the fold change obtained from the HSF Chamber exposures. As shown in Figure 5, no differences were observed at each condition in the CCES when compared to the HSF Chamber. Finally, we evaluated LDH release into the basolateral medium 24 h post-exposure for all exposure conditions to determine if cytotoxicity occurred. Baseline levels of LDH release in the incubator controls were ~8.4% of total. As shown in Figure 7, no difference in LDH release was observed among the exposures to acrolein using either flow rate in the CCES and the HSF Chambers. These results suggest that the flow rates tested under our conditions do not induce cytotoxicity due to cell desiccation or stress resulting from the increased air flow.

Discussion

We sought to miniaturize our existing ALI exposure system (HSF Chambers) by developing and optimizing a new *in vitro* exposure system (CCES) for testing of volatile chemicals. The CCES accommodates 24-well cell culture plates containing Transwell inserts to permit the exposure of cultured cells at ALI conditions. Previously, similar ALI *in vitro* exposure systems have been developed and mostly characterized for their particle deposition efficiency (Aufderheide and Mohr 2000; Phillips et al. 2005; Savi et al. 2008; Stevens et al. 2008; de Bruijne et al. 2009; Lenz et al. 2009; Volckens et al. 2009; Aufderheide et al. 2011). These systems, however, have not been tested with acrolein, thus a direct comparison cannot be made. Additionally, most of these systems require the porous membrane inserts to be transferred from their multi-well cell culture plates into custom-designed wells inside each system. This results in extensive handling of the porous membrane inserts, cell culture medium changes from multi-well culture plates to exposure system and back to multi-well plate after exposures, and cleaning/maintenance to sterilize custom-designed wells. Thus, researchers are limited to generating low-throughput data due to the laborious process, as well as the complexity of generating test atmospheres.

The key advantage of our new CCES is that by accommodating the 24-well plates, we minimized the handling of Transwell inserts and improved turnaround times between back-to-back exposures because no maintenance or cleaning is needed between testing. The CCES is housed in a temperature-regulated enclosure maintained at 37°C along with a humidification system that conditions the sampled flow to >80% RH to better maintain cell integrity. Moreover, the flow rate was optimized and the efficacy of this new system was compared to incubator chambers that have been used widely at EPA's HSF for the past 26 years. The HSF Chambers served as our benchmark to help optimize and improve the sensitivity of the CCES. We showed that operating the CCES at a flow rate of 25 mL/min/well provided similar biological responses to those resulting from HSF chambers, thus demonstrating the utility of the CCES. The slightly lower sensitivity of the CCES could be a result of the lower flow rates used in the overall system compared to the HSF Chamber flow

rates. Since the CCES has nozzles guiding the air and test gas directly over the cells, increasing the flow rate can lead to adverse effects, such as cell desiccation; thus, our current flow rate of 25 mL/min/well could be the maximum the cells can handle; more testing is needed to determine if the flow rate can be increased.

We have previously demonstrated using a modified VITROCELL® 6 CF system that with the appropriate temperature and humidity we can eliminate the cytotoxic effects resulting from control clean air exposures of 1 h in BEAS-2B cells (Zavala *et al.*, 2017). In this study, we have used the CCES to expose cells to clean air for up to 2 h without inducing adverse effects to the cells and demonstrate that conditioning of the sampled air is critical for conducting ALI exposures. Further testing is required to determine the maximum time cell cultures can be exposed without comprising cell viability during sham (control air) exposures. It is expected that as air flow rate is increased, the maximum exposure time could be reduced.

We optimized the design and operating conditions of the CCES by conducting characterization tests using O₃ as a test gas for a fluorescence-based method. In this method, we measured the reactivity of O₃ on an ITS-impregnated filter placed inside each well. In this method, no cells were used; we simply measured a chemical reaction. Typically, similar *in vitro* exposure systems in a 24-well format use flow rates as low as 2 mL/min/well. Here, we tested a flow rate of 5 mL/min/well as well as 25 mL/min/well because we postulated that a much higher flow rate would deliver the test gas more effectively. Our results confirmed our hypothesis that the higher flow rate would be more effective because it resulted in a higher fluorescence, meaning that more of the chemical reached and reacted with the ITS-impregnated filter. Nonetheless, such data are a measure of only a chemical reaction on the surface of the membrane and not of biological responses.

To demonstrate the effectiveness of this method to predict a possible biological response, we exposed HBECs to 0.3 ppm acrolein for 2 h using 3, 5 and 25 mL/min/well flow rates. We assessed *HMOX1* and *IL-8* mRNA levels, as well as LDH release, at various time points and determined that this concentration of acrolein elevated *HMOX1* mRNA when measured 1-h post-exposure. This biological result paralleled that of the chemical (fluorescence) result, i.e., the higher flow rate induced a significantly higher response. These results lead us to conclude there are two important factors to consider: (1) At the lower flow rates, the residence time (between the air entering the distribution manifold and reaching the cells) is long enough to permit test gases to react with the surfaces, scrubbing out the test gas; so, by the time an 'air parcel' reaches the cells, the concentration of the test gas is likely diminished even after all sample lines and system has been conditioned. (2) Higher flow rates created more turbulence and better mixing above the cells, therefore increasing the rate of diffusion for the gases. This is a critical finding because, as mentioned earlier, similar exposure systems use flow rates <10 mL/min/well, which would likely require the use of much higher starting concentrations of chemicals to induce biological effects. Because the CCES was machined out of stainless steel, a relatively inert material, we believe the turbulence created by the higher flow rate over the cells is the dominant factor responsible for delivering the test gas more effectively to the cells. Additionally, using Fick's Law of diffusion, we estimated the mass flux of acrolein to the cell surface to ensure that the

acrolein delivered at the various flow rate conditions was not a factor in the observed chemical and biological measurements observed.

Three other studies that we are aware of have exposed cells to acrolein at ALI, however, the exposure conditions and/or cell culture model are different, thus difficult to make a direct comparison. In one study, A549 cells were exposed to acrolein for 4 h to 0.23, 0.63, 1.0, 1.47, and 2.37 ppm using the Gas In Vitro Exposure System (GIVES) (Lin et al. 2014). Here, no changes in cytotoxicity (LDH) were observed at 0.23 or 0.63 ppm, however at 1 ppm a ~3-fold increase in LDH and ~2-fold increase IL-8 protein (measured in the basolateral medium) were observed when assessed 9 h post-exposure. Similarly in a second study using a similar GIVES setup, A549 cells exposed to acrolein for 4 h to 1.0 ppm yielded a ~3-fold increase in both LDH and IL-8 protein (measured in the basolateral medium) when assessed 9 h post-exposure (Ebersviller et al. 2012). In the GIVES setup, temperature is regulated at 37°C and RH in the exposure chamber is ~40%; RH is less critical in this setup as air circulates over the 8 L chamber, and not directly guided to each insert. In the third study, primary bronchial epithelial cells were exposed to 0.05, 0.1, 0.2, and 0.5 ppm acrolein for 30 minutes using a modified 20 L glass desiccator as an exposure chamber (Dwivedi et al. 2018). There, exposures were limited to 30 minutes because at 0.2 and 0.5 ppm reductions in cell viability of $80\% \pm 5\%$ and $70\% \pm 5\%$ (mean $\pm$ SD), respectively, were observed. During exposures, cells were maintained at 22°C with RH at 45–52%; the lack of temperature regulation and low RH likely caused the reductions in viability. *HMOX1* was assessed 6 h post-exposure and a 5.5-fold increase was observed. No changes in IL-8 protein (measured in the basolateral medium) were observed at 8 or 24 h. Our current study seems to agree with the results observed previously (Dwivedi et al. 2018) where gene-expression of *HMOX1* is altered in primary bronchial cells but no changes to IL-8. When comparing our current study with studies using A549 cells, our results would have been more strongly supported if we had analyzed the basolateral medium for IL-8 protein release 24 h post exposure as opposed to only the IL-8 transcript levels using RT-PCR.

Our *in vitro* exposure system facilitates the exposure of cells to volatile chemicals in a more effective manner than submerged exposures. Its small footprint and transportability will permit the deployment of various units in multiple labs to achieve higher throughput by scaling-up the number of simultaneous exposure studies possible. We demonstrated the utility of our miniaturized system by comparing our results to those obtained from a well-established exposure system at the U.S. EPA (HSF Chambers). When developing new *in vitro* exposure technology, extensive characterization tests are needed to understand fully the limitations of the system and the optimal operating conditions. Although we have presented results using a 24-well format, the CCES is a versatile system that can easily be modified and adapted to other exposure conditions. For example, a unique feature of the CCES allows the removal of the two distribution manifolds, permitting the exposure of cells to 6 different concentrations at once (in groups of 4 wells per concentration); a future study will demonstrate this capability. Another unique feature is that the top cover containing the nozzles is interchangeable to accommodate a 6-well format, while maintaining the same base module. The versatility and effectiveness of the CCES makes this a promising new tool for future *in vitro* toxicology studies to aide in EPA's mission of sustainability to help refine,

reduce, and replace (The 3 R's) animal models used to study potential health effects of airborne contaminants.

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Figure 1.

The cell culture exposure system (CCES). The inlet port provides the exposure atmosphere to the two inlet distribution manifolds. Total air flow is separated, and the resulting air flow rate to each inlet mixing manifold is reduced by half. Each manifold contains 3 delivery ports that guide the air flow into an annular space where the flow is then split into 4 nozzles. The individual nozzles sit centered 2 mm above each Transwell inserts resting on a 24-well cell culture plate. Therefore, each Transwell insert is under a slightly positive pressure to allow for continuous delivery of fresh material. The exhaust port is under a slight negative pressure and pulls spent exposure atmosphere from each Transwell insert. The static pressure in the entire closed system is maintained under a slight positive pressure.

Figure 2.

Experimental setup of two CCES units mounted on an enclosure cart. A heater and fan help regulate temperature, whereas diffusion humidifiers help regulate RH levels. One CCES unit is used for filtered air exposures (sham exposure) and the other unit is used for test gas exposures.

Figure 3.

Fluorescence profile of ITS-impregnated filters exposed to 125 ppb ozone for 10, 30, and 60 min. An excitation wavelength of 260 nm was used with an emission wavelength scanning from 350–500 nm in 10-nm increments.

Figure 4.

Fluorescent intensity (excitation = 260 nm, emission = 410 nm) resulting from ITS-impregnated filters exposed to 125 ppb O_3 for 1 h ($n = 3$). A significant increase was observed when the flow rate was increased from 5 to 25 mL/min/well. Additionally, a significant increase is observed in from the HSF Chambers when compared to the CCES at both flow rates. Bars represent average fluorescent intensity $\pm$ SD. Asterisks represent a p-value of <0.001 (***) determined using a one-way ANOVA with Tukey's post test.

Figure 5.

Comparison of *HMOX1* transcript abundance at 0, 1, and 24 h post exposure to 0.3 ppm acrolein ($n = 3$) in either the HSF Chamber or at different flow rates (3, 5 or 25 mL/min/well) in the CCES. *HMOX1* transcript levels were normalized to corresponding beta-actin transcript levels. Bars represent average fold change $\pm$ SD (two-way ANOVA with a Bonferroni post test). An asterisk represents a p-value of <0.05 (*) and asterisks represent a p-value of <0.001 (***) when compared to the matched HSF Chamber post exposure duration.

Figure 6.

Comparison of *IL-8* transcript abundance at 0, 1, and 24-hours post exposure to 0.3 ppm acrolein ($n = 3$) in either the HSF Chamber or at different flow rates (3, 5 or 25 mL/min/well) in the CCES. *IL-8* transcript levels were normalized to corresponding beta-actin transcript levels. No differences in response between the HSF Chamber or the CCES at various flow rates were observed. Bars represent average fold change $\pm$ SD.

Figure 7.

Comparison of LDH release 24-hours post exposure to 0.3 ppm acrolein ($n = 3$) in either the HSF Chamber or at different flow rates (3, 5 or 25 mL/min/well) in the CCES. No differences in response between the HSF Chamber or the CCES at various flow rates were observed. Bars represent average percent LDH release $\pm$ SD.

Table 1.

Primer and probe sequences for *IL-8*, *HMOX1*, and *ACTB*.

Gene		Sequence (5' – 3')
<i>IL-8</i>	Forward	TTGGCAGCCTTCCTGATTTC
	Reverse	TATGCACTGACATCTAAGTTCTTTAGC
	Probe	(FAM)-CCTTGGCAAACTGCACCTTCACACA-(ZEN)
<i>HMOX1</i>	Forward	GAGGGTGATAGAAGAGGCCAAGA
	Reverse	GGTCAGCAGCTCCTGCAACT
	Probe	(FAM)-TGCGTTCCTGCTCAACATCCAGCTC-(ZEN)
<i>ACTB</i>	Forward	CTGGCACCCAGCACAAATG
	Reverse	GCCGATCCACACGGAGTACT