**Dear Dr. Steindel:**

**Enclosed please find our responses to the reviewers’ comments and the revised manuscript. We would like to thank the reviewers for their thoughtful critiques of the manuscript. The comments and recommendations of the reviewers have helped us improve the presentation and clarity of the method. Please find below response to the individual reviews. Changes to the manuscript were incorporated in red font. We hope that our specific responses to the reviewers adequately address any concerns. Thank you for considering the revised manuscript.**

**Editorial comments:**  
Changes to be made by the Author(s):  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been thoroughly proofread, ensuring there are no spelling or grammatical issues.

2. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

The summary has been rephrased to clearly describe the protocol and applications. “Here, we present a protocol to perform portable cellular aerosol exposures and measure cellular response. The method uses cells grown at the air-liquid interface, mimicking *in vivo* physiology. Cellular response to copper nanoparticles was observed as oxidative stress through reactive oxygen species generation and cytotoxicity as lactate dehydrogenase release.”

3. Please rephrase the Abstract to more clearly state the goal of the protocol.

The abstract has been re-written to more clearly state the goal of the protocol. “This protocol introduces a new *in vitro* exposure system, capable of being worn, including its characterization and performance. Air-liquid interface (ALI) *in vitro* exposure systems are often large and bulky, making transport to the field and operation at the source of emission or within the breathing zone difficult. Through miniaturization of these systems, the lab can be brought to the field, expediting processing time and providing a more appropriate exposure method that does not alter the aerosol prior to contacting the cells. The Portable *In Vitro* Exposure Cassette (PIVEC) adapts a 37 mm filter cassette to allow for *in vitro* toxicity testing outside of a traditional laboratory setting. The PIVEC was characterized using three sizes of copper nanoparticles to determine deposition efficiency based on gravimetric and particle number concentration analysis. Initial cytotoxicity experiments were performed with exposed lung cells to determine the ability of the system to deposit particles while maintaining cell viability. The PIVEC provides a similar or increased deposition efficiency when comparing to available perpendicular flow *in vitro* exposure devices. Despite the lower sample throughput, the small size gives some advantages to current *in vitro* ALI exposure systems. These include the ability to be worn for personal monitoring, mobility from the laboratory to the source of emission, and the option to set-up multiple systems for spatial resolution while maintaining a lower user cost. The PIVEC is a system capable of collecting aerosols in the field and within the breathing zone onto an air-interfaced, *in vitro* model.”

4. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:  
1.1.1: Please specify the size of the tubing and connectors.

1.1.1 Make sure to use new or well cleaned with 70% ethanol ¼” inner diameter tubing and ¼” outer diameter connectors for system assembly.

1.1.2: Please specify the temperature and humidity suggested for storing the test materials.  
A schematic of the system and setup as Figure 1 would greatly aid in step 2.1 of the protocol.

1.1.2 Store test materials including filters, PIVEC components, tweezers, and particle powders in a well-controlled environment, with respect to temperature and humidity, for at least 24 hours prior to experiment. The temperature should be near room temperature, approximately 20 C, with relative humidity less than 35%. This is very important to achieve repeatability between experiments.

3.1.1: Please specify the pore size of the filters used.

3.1.1 Keep 1.00 µm pore glass fiber filters under low humidity conditions for at least 24 hours prior to pre-exposure measurements. Weigh the filter three times and record the filter weights. Place the filter in a cell culture insert.

4.1.2: How to obtain the mass-based deposited dose?

4.1.2 Use administered mass, madmin, and mass-based deposited dose determined in 4.1.1, mdep, to calculate the mass-based deposition efficiency, ηm, for experiment.

5. Please include single-line spaces between all paragraphs, headings, steps, etc.

The manuscript has been revised to include the single-line spaces throughout.

6. After you have made all the recommended changes to your protocol (listed above), 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.

Approximately two pages of the protocol has been highlighted in yellow. This covers steps 2 and 5 to show the set-up of the system and exposure of cells to a dry aerosol.

7. 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.

Steps 2 and 5 were highlighted. This will show the set-up of the aerosol and PIVEC exposure system and exposure of cells within the PIVEC to a dry aerosol.

8. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

All steps within the sections have been highlighted.

9. Please number the figures in the sequence in which you refer to them in the manuscript text. Currently Figure 2 (line 164) is mentioned before Figure 1.

Thank you for bringing this to our attention. The figures have been renumbered and figure captions adjusted accordingly. Line 164, Lines 292-296.

10. Lines 310-311: Please remove commercial language including trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. For example: Cultex CG, Vitrocell.

The commercial language have been removed from the manuscript.

From the Introduction: “Perpendicular flow, used by a microsprayer,9 MINUCELL,12 NACIVT,13 and commercial ALI systems<sup>10,11,14</sup>, adds the impaction of particles within the deposition region.”

From the Representative Results: “Compared to similar systems throughout literature, including the MINUCELL14,60 and NACIVT61 systems, the deposition efficiency of the PIVEC over the range of particle sizes tested is comparable or increased over reported values, observed in Figure 4.”

11. Discussion: Please describe critical steps within the protocol.

The discussion of critical steps within the protocol has been more explicitly revisited in the Discussion section from the Results section. “Deposition can be increased by changing the flow rate and exposure duration. This may also influence the cell viability with the potential of drying out the cells. The conditioning of aerosols is often performed to compensate for physiological attributes such as body temperature and humidification in the airways. Increased humidity, over 50%, mimics inhaled air and decreases cell death due to vehicle exposure.63 When temperature and humidity are not well controlled, the cellular response can be influenced. By decreasing the flow rate, additional particles of all sizes will deposit, increasing the deposition. Exposure duration is proportional to deposition, allowing more particles to deposit over an extended experimental period. Conditioning of the aerosol is important when increasing the exposure duration so that the cells do not dry out which can affect biological responses. Additionally, by conditioning the filters as described in Step 1 of the protocol, the deposition efficiency can be accurately determined.”

**Reviewers' comments:**  
  
Reviewer #1:  
  
Manuscript Summary:  
The authors have described a portable in vitro exposure sampling method.  
  
Major Concerns:  
None  
  
Minor Concerns:  
Even the system is considered small and portable this system still requires SMPS/OPS which are considerably large instruments for the setup.  
Revisions needed in introduction, methods, results, discussion, and references sections. See below  
  
Introduction:  
Authors Introduced the subject well. . The authors describe the disadvantages of other ALI systems due to their size, how it affects the data (line 97..). Including more details how the PIVEC system overcomes the described issues can be more meaningful to the reader. The authors can include some further information comparing the PIVEC to other ALI systems comparing not only particle exposure but also gaseous exposures and measurements. Visualizing the PIVEC system is difficult without mentioning the dimensions and weight, including these may help the reader.

We thank the reviewer for their insight. Additional information has been added to the Introduction section discussing the ability of the PIVEC to overcome described issues, including the dimensions and weight of the system. Additional comparisons between the PIVEC and other ALI systems is found in the Results and Discussion sections. “The PIVEC is smaller than current systems, with an external surface area of approximately 460 cm2 and weighing only 60 grams, with thermal and humidity control incorporated into the system allowing for a highly portable device. The decreased size and weight allows the system to be worn or taken to the source of exposure, permitting direct sampling.”

Protocol:  
Well written. Figure 1 is not very clear. Increasing the clarity of the pictures and labeling the pictures arrows may be helpful to the reader. For 6 and 24 well plates, can cells in actual ALI conditions be used? If so how are the ALI conditions achieved in this system? Include some details (line 198).

Thank you for helping us clarify this. For both 6 and 24 well plates, cells in the ALI conditions can be used. To aid the reader in understanding how ALI conditions are achieved, an additional section, 5.1 Culture Cells at Air-Liquid Interface, has been added to the protocol.

**“5.1 Culture Cells at Air-Liquid Interface**

5.1.1 Lift cells from culture flask by adding trypsin-EDTA, 3 mL for a T75 flask or 1 mL for a T25 flask, and incubate for 5 minutes at 37°C. Add complete media to flask and rinse flask wall with cell suspension to maximize the recovered cell number. Transfer cell suspension to a sterile 15 mL conical tube then centrifuge cells at 1200 rpm for 3 minutes.

5.1.2 Remove supernatant containing trypsin-EDTA and resuspend cell pellet in 10 mL of complete media. Remove 10 µL of cell suspension and add to hemocytometer. Count cells in hemocytometer to determine the concentration and total number of cells.

5.1.3 Add complete media to each well in well plate, for 24 well plate add 0.5 mL to each well. Place cell culture inserts in wells. Seed cell culture inserts on apical side at a cell density near 1X105 cells/cm2 for cell types that grow at a rate near doubling per day. Note: Cells with a slower growth rate can be seeded at an increased cell density.

5.1.4 Add complete media to apical side of transwell to reach final volume, for 24 well plate final volume is 0.25 mL.

5.1.5 Culture for 7 days in submerged conditions, replacing media every 1-2 days. After 7 days, remove apical media and culture for at least 1 day in ALI conditions, replacing only the basolateral media.”

Results:  
The deposition efficiencies are still very low. Can this be improved? How uniform is the deposition between wells (6 or 24)? Are there any data to show equal deposition between the wells?

The reviewer is correct that the deposition efficiencies are still low for an ALI exposure system. The deposition efficiency can be improved within the system and will change for different aerosol compounds. The issue of uniform deposition is also addressed within the revised manuscript. “The deposition in the 24 wells is very uniform over the insert, however, deposition in the 6 well design is lacking uniformity as most of the particles deposit near the center of the insert.” … “The deposition efficiency within the PIVEC can be improved through minimizing losses to the system using electrostatic dissipative or conductive plastic or similar material to design the PIVEC.”  
  
Figure 3: Clearly label a, b, and c.

The figures are labeled in the upper left corner of the chart.

Figures 5 and 6: Show statistical comparisons.  
Figure legends: Include statistical comparisons and p values for the figures.

Thank you for helping us to clarify the statistical comparisons of these measurements. The manuscript has been adjusted to reflect these changes. “Figure 5. Cellular Response to Copper Nanoparticles Post-Exposure (PE). For all measurements, n=3 and p<0.05. A) Oxidative Stress determined using the DCFH-DA Assay. B) Cytotoxicity determined using the LDH Assay.”  
  
Include a section on statistical methods.

Thank you for the suggestion. A section on statistical methods used has been included in the updated protocol.

**“6 Statistical Methods**

6.1 Analyzation of Biological Assay Data

6.1.1 Report ROS production as the fluorescence intensity increase of treated cells relative to baseline measurements. Report LDH activity as the fluorescence intensity increase of treated cells relative to untreated cells.

6.1.2 Perform single factor ANOVA to determine statistical differences between data sets. Where appropriate, perform student t-tests at a value of significance of 0.05. Report data as the mean ± standard deviation of at least three exposure measurements.”

Discussion section: The discussion section very short. Consider expanding based on the results.

The discussion section has been expanded with relationships of the deposition and exposures within the PIVEC to alternative perpendicular flow systems.

References: Too many references, maybe reduce by including only the most relevant references.  
  
The number of references cited has been reduced to 49 from 65.  
  
  
Reviewer #2:  
  
Manuscript Summary:  
The authors describe a portable system to expose cells at the air-liquid interface to detect cytotoxic effects of aerosols for personal monitoring in the breathing zone. In general, this is a challenge which has been implemented.  
They used three different sizes of Cu nanoparticles to generate aerosols, to determine the deposition efficiency and to expose cells.  
In summary, the manuscript is well written and the well-structured figures clearly demonstrate the results.  
  
Major Concerns:  
  
The data of the aerosol characterization (mass concentration, number concentration) are missing.

Thank you for bringing this to our attention. The SMPS and OPS measurements for each aerosol are included now as Figure 3. “Figure 3. Particle number concentration of copper nanoparticle aerosols. A) SMPS measurement. B) OPS measurement.”

Which method was used to humidify the aerosol?

The aerosol was humidified using a porous, wetted tube. This is now reflected in the manuscript. “The temperature was maintained through the use of a battery-powered resistive heater and the aerosol humidified using increased natural humidification through a porous, wetted tube.”  
  
Page 9, line 320: the flow rate over the cells is 0.5 LPM. This flow seems extremely high for the small inserts (24-well plate inserts) in comparison to the flows used in other studies using CULTEX, MINUCELL, NACIVT or VITROCELL systems, which use flows between 0.005 and 0.1 LPM for bigger inserts (6-well plate inserts). Such a high flow rate would probably damage the cells by shear stress.

The reviewer brings up a valid point that the increased flow rate may damage the cells via shear stress. To address this, the following line has been added to the manuscript “Alternative perpendicular flow exposure systems use between 0.005 LPM and 1.5 LPM for a sustained exposure period whereas this method uses a moderate flow rate during a rapid exposure.”

Minor Concerns:  
  
Which types of cells were used to create the data in Fig. 5?

Thank you for bringing this to our attention. The cells used in this study was the A549 cell line, an alveolar epithelial cancer cell commonly used in inhalation toxicology. “A549 cells, an alveolar epithelial carcinoma cell line, were exposed for 10 minutes to varying sizes of copper nanoparticles at a flow rate of 0.5 LPM.”  
  
Page 3, line 79: It should be specified that only primary lung cells are able to generate a differentiated cell monolayer.

The reviewer is correct that only primary lung cells will generate a differentiated cell monolayer. Cell lines, however, will produce a polarized barrier that may reflect some *in vivo* characteristics depending on the line and method of culture. “Lung cells grown at the ALI have the ability to generate a polarized barrier layer,21–24 which produces physiological traits that resemble the *in vivo* lung epithelium, including mucus and surfactant production in specific bronchial or alveolar cell lines, cilia beating,22 tight junctions,22,25 and cell polarization.21”  
  
Page 5, line134: "… using in vitro exposures with confirmation in vitro." I guess that one of the terms "in Vitro" should be "in vivo"

Thank you for pointing this out. The reviewer is correct and the appropriate change has been made. “Out of ten nanosized metal oxides tested, six tested as active (titanium oxide, zinc oxide, and four different cerium oxide) using exposures *in vitro* with confirmation *in vivo*.“  
  
Only few tube materials are suitable for conducting aerosols. Therefore, the material of the tubings should be known.

The reviewer is correct that only few tube materials are suitable for conducting aerosol experiments due to potential interactions and losses to the tube. In this study, conductive tubing was used to decrease these losses.   
  
Page 9, line 323: "Cytotoxicity and oxidative stress were observed within the first twenty-four hours of exposure." This is misleading. In Line 319 it is written that the exposure was for 10 min and here for 24h?

Thank you for bringing this to our attention. The exposure was for 10 minutes and biological endpoints were observed within 24 hours post-exposure. The manuscript has been amended to make this clearer. “Cytotoxicity and oxidative stress were observed within the first twenty-four hours post-exposure.”  
  
Page 9, line 325: In the text it is said that figure 5b shows results of "4h and 24h post-exposure", however figure 5 does not show the 24h results. Later on it is said that the 24h viability results are not shown, why not? This would be interesting for the reader. This could also show if exposure of cells themself was cytotoxic or the exposure to the nanoparticles.

Thank you for helping us to clarify this in the text. The 24 hour viability results were not shown as there was significant variability between experiments. However, within this study, it does appear as though the exposure itself produced some cytotoxicity.

“Cytotoxicity was measured using the release of lactate dehydrogenase (LDH) from damaged cells immediately, 4 hours, and 24 hours post-exposure. There was no significant toxicity from copper nanoparticles below 1.62 mg/cm2 within 4 hours of exposure, Figure 6b.”  
  
Figure 5: please explain the abbreviation "PE" in the legend.

The manuscript has been adjusted to reflect this addition. “Figure 6. Cellular Response to Copper Nanoparticles Post-Exposure (PE). A) Oxidative Stress determined using the DCFH-DA Assay. B) Cytotoxicity determined using the LDH Assay.”