Vinciane Saint-Criq
Institute for Cell & Molecular Biosciences
University Medical School
Newcastle University
Newcastle upon Tyne, NE2 4HH, UK

Dear Colleagues,

Re: JoVE59815 "Real-time, semi-automated fluorescent measurement of the Airway Surface Liquid pH of primary human airway epithelial cells "

We are grateful to the reviewers and editors for their interest in our paper. We have addressed every one of the editors and reviewers comments below. We believe the manuscript is improved and hope that the manuscript will now be acceptable for publication.

In keeping with your email, we have addressed each comment separately by denoting comments from your email as C1, C2 etc, including text from the email in italics. Our responses are marked R1, R2 etc in blue normal font.

We also attach our revised manuscript, with tracked changes, as requested and the revised Figures 1, 2 and table of materials.

Editorial comments:
Changes to be made by the author(s):
C1. 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.
R1. We have thoroughly proofread the manuscript for any typographical and grammatical errors.

C2. As some authors are affiliated with UK institutions, can you please check whether open access is required by your funding agencies?
R2. This has now been checked and open access is not required.

C3. Please shorten the title to be more concise if you are able.
R3. The title has been shortened to a certain extent as we feel some important information would be missing if we shortened it further. It now reads: “Real-time, semi-automated fluorescent measurement of the Airway Surface Liquid pH of primary human airway epithelial cells”.

C4. 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. You may use the generic term followed by “(Table of Materials)” to draw the

readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Transwells, pHrodo, Microsoft excel, etc.
R4. All trademark symbols have been removed and generic terms now replace the commercial names.

C5. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
R5. The Protocol has been revised and any personal pronouns removed.

C6. 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.
R6. The comments below have been addressed and more details have been added to the protocol.

C7. 1.2: Please list an approximate volume to prepare.
R7. The volume of solution has been added to the text

C8. 1.3: This step is unclear. Do you mean the basolateral medium used to grow primary hAECs? Please also provide its composition.
R8. We have now specified that it is the differentiation medium used for cell growth and differentiation. The protocol has been established by another group (cited in section 1.1 and now 1.3) and its composition is provided in the two methods publications cited.

C9. 1.4: What is KRB? Please also provide its composition.
R9. The abbreviation and meaning of HCO_3^- containing Krebs buffer solution (HCO_3^- KRB) has now been added in section 1.2.

C10. 1.5: Please describe how to remove the apical wash.
R10. Further details about how to remove the apical wash have been added to 1.5

C11. 1.6: Is medium added in this step?
R11. No medium is added apically at this step, as the cells need to be maintained at air-liquid interface. This has now been specified on section 1.5.

C12. 2.9: Please provide the composition of the dye mix.
R12. The composition of the dye mix is given in section 2.8. As there are 3 notes between 2.8 and 2.9, the section where the composition is given is now specified.

C13. 3.1-3.4: As these steps are the same as 2.1-2.4, they can be simplified, for example: "Repeat steps 2.1-2.4 to prepare the plate reader".
R13. This has now been changed.

C14. 3.7: How to start, by clicking a button?
R14. More details on how to start the reading have been added in section 3.7

C15. 3.9: When and why is the plate put back on the tray?
R15. The plate is put back on the plate reader tray after the addition of agonists/drugs to the cells to monitor pH changes induced by these molecules. This is now explained in section 3.6

C16. 4.5: Please specify the specific step where these parameters are described.
R16. This has now been added: "Set up the plate reader with the same parameters as described previously, but with no CO_2, as in step 3.2."

C17. 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. Notes cannot usually be filmed and should be excluded from the highlighting.
R17. This has been checked throughout the protocol text.

C18. 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.
R18. This has been checked throughout the protocol text.

C19. Figure 1: Please include a space between numbers and their temperature unit (37 °C). Please remove commercial language (pHrodo/AlexaFluor® and replace with generic terms.
R19. A space has been added between numbers and their temperature unit on Figure 1

C20. Figure 2: Please abbreviate liters to L (L, mL, µL) to avoid confusion.
R20. This has been changed on Figure 2.

C21. Table of Materials: Please remove trademark (™), registered (®) and copyright symbols. Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.
R21. These symbols have been removed and items sorted in alphabetical order.

C22. References: Please do not abbreviate journal titles.
R22. The journal names have now been changed.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

C23. The authors presented a detailed and practical protocol to a semi automated fluorescent assay that required a lot of calibration in order to be reproducible.

The guidance at every step and the explanation given to better understand the basic controls and more defined calibrations allow this protocol to be an easy hand for anyone in the field who want to start ASL pH measurement.

It would be interesting to present this guide as a table reporting all the control to be done in order to implement the experiment.

R23. We thank reviewer 1 for their helpful comments. However we are unsure about the benefit of a table compared to the details that are already present in Figure 1, in which important controls are highlighted for the entire process of the assay.

Major Concerns: none

Minor Concerns:

C24. When calibrating the background of the plate and actually measurement of the fluorescence of the plate, the authors are not cleared about keeping the lid or not on the plate. This raises a minor concern:
According to the authors, the background calibration requires up to 10 min in the reader, would it be possible to get contamination form this step, knowing how sensitive are these cells?
R24. The tissue culture plate lid is placed on the plate at all times, apart from when adding drugs/changing the medium – which are performed in a tissue culture laminar flow hood. A note specifying this has now been added in section 2.5.

C25. The assay aim is to measure pH in the ASL. As the authors described it in the limitation, it is not possible to have the same ASL volume for all the conditions
The ASL depth (hydration) is however significantly different between CF and non CF cells, which does not allow to study CF cells versus non CF cells, but definitely would be suitable to screen for correctors and potentiators on Cf cells.
R25. Although it is true that ASL volume will affect fluorescence readings and therefore the calculated pH – as presented in figure 2 - comparison between CF and non-CF cultures can be performed because of the in situ calibrations that are performed on each set of donor cultures individually.

Reviewer #2:

C26. Manuscript Summary:
The measurement of airway surface liquid (ASL) pH is notoriously difficult. A number of different methods have been developed to measure ASL pH, but the reliability and reproducibility of these different methods have often come into question and conflicting findings have further fueled the controversy as to whether there exist differences in ASL pH between CF vs. non-CF airways. In this manuscript, Saint-Criq et al describe a new method to measure ASL pH using a cell-impermeant pH-sensitive fluorescent dye and a standard plate reader that rigorously controls for different volumes and dye concentrations to more reproducibly measure ASL pH. Using this method, the authors demonstrate that the difference in ASL pH between CF vs. non-CF airways is not significant and provide robust data

to support this conclusion. The authors further show that "expected" changes in ASL pH, in this case through forskolin stimulation, can be readily detected in real-time experiments. The manuscript is well written and the protocol is straightforward, providing sufficient detail for readers to implement these measurements. Although there are limitations to the method, these are addressed by the authors.

R26. We are grateful to Reviewer 2 for such a positive comment, and for raising a number of interesting points that we have addressed below.

Major Concerns:

C27. 1) ASL pH can vary greatly from donor to donor. It would be interesting to see the variability in ASL pH in ALI cultures from the same donor.

R27. We agree with Reviewer 2 concerning the inter-donor variability although for the results obtained on cells from the 3 non-CF donors, variability was small. ASL pH was 6.84, 6.89 and 6.91 and standard deviation were 0.21, 0.29 and 0.20, respectively. Although we believe this is a fair point, we do not believe that addition of these data would add valuable information to this methods paper.

C28. 2) Mucus remains a problem when measuring ASL pH which is a point the authors concede in the manuscript. Indeed, CF ALI cultures tend to have much more mucus that non-CF cultures. How do the authors control for this and what effect is this expected to have on the measurement of ASL pH?

R28. It is true that CF cultures usually secrete a very sticky mucus. However, we have optimized the wash technique and the 20 min wash at 37 °C removes most of the mucus.

C29. 3) The glucose level in the basolateral media can have a significant impact on ASL pH. Have the authors measured ASL pH using different glucose concentrations in the basolateral media? The importance of controlling for glucose levels in the basolateral media should at least be stated in the methods.

R29. We are aware that glucose concentration can affect ASL pH as described by Garnett et al. In our experiments we have kept glucose constant and as suggested by Reviewer 2, we have now stated the importance of the basolateral glucose content in a note in section 1.3 of the protocol.

C30. 4) From a technical standpoint, how easy it to reliably deliver 3 µL of dye mix to the apical surface of the cells?

R30. As for every experiment that is performed on epithelial cells at air-liquid interface, extra-care needs to be taken in order to avoid scraping the epithelial surface. However, this technique does not require specific skills and can be mastered with a fairly standard amount of practice.

Minor Concerns:

C31. 1) What are the CFTR mutations from the CF donors? This should be stated.

R31. This has now been added to the protocol text in section 1.1

C32. 2) Age may also have an impact on ASL pH. What are the ages of the donors? This should also be stated

R32. This has now been added to the protocol text in section 1.1 although the age is unknown for one of the CF donors.