**Respond to Reviewers' Comments**

**Reviewer #1**

*Summary:*   
The manuscript by McGovern et al describes the protocol for analyzing respiratory mechanics using forced oscillation technique. Overall, the manuscript is well written, organized, and adequately detailed. However, this reviewer has concerns over the title of the manuscript; specifically, the exclusion of acknowledgement that the specific protocols being described derive exclusively from Scireq.

*Major Concerns:*

1. This reviewer is aware that the flexiVent/flexiWare system is widely used, especially with forced oscillation technique, in the field of respiratory research. Because other ways of FOT exist, it is worth mentioning in either the title or abstract that this manuscript is tailored to use of a particular software.

The authors identified the system used in the abstracts (long & short).

2. Is there any information the authors could include regarding the room temperature stability of methacholine? Since the compound itself is unstable and must be stored at very low temperature, does it become less effective with respect to animal response over hours of experiment time?

The authors added a reference on the stability of methacholine over a period of 9 months. We also calculated from that data that methacholine degradation at room temperature over the course of a day would be minimal.

3. "Execute a deep inflation... The recorded volume and pressure traces should also be smooth with no signs off offset?" Can the authors provide visual representations of what should be considered acceptable/unacceptable? What would the curve look like with a spontaneous breath from the animal, and how would it translate to downstream analysis? This reviewer feels that it would better communicate the information to the reader.

The authors added a representative screenshot of what would be considered “acceptable” traces following a deep inflation of the lungs. The question of spontaneous breathing effort was illustrated in a separate figure using a stepwise pressure-volume curve. The impact of spontaneous breathing effort (excluded datasets) was added in the discussion.

The visual representation will also be available in the video.

4. "Verify the absence of?Observe the pressure signal traces?" Same comment as 3.

Please see reply to previous comment.

5. Can the authors mention why use of Quick Prime-3 would be preferred over Quick Prime-8?

The authors mentioned in the representative results section that the Quick Prime-3 was preferred over the Prime-8 because of its shorter duration (3 vs 8 seconds) in order to shorten the apneic period, minimize the effect of the perturbation on blood gases and obtain a better resolution of the response.

6. Are there any aspects of the protocol that may change with the strain/age of mouse? (i.e. diameter of endotracheal cannula, dose of anesthesia, "normal" baseline resistance values)

The authors referenced some work in the discussion related to measurements in various mouse strains and developing mice.

What is important in terms of the endotracheal cannula is its resistance relative to the subject’s resistance. We added in the discussion that increasing the cannula inner diameter and / or shortening its length should reduce the resistance of the cannula.

Finally, the anesthetic regimens mentioned in Table 1 include various mouse strains.

7. How does the length of the endotracheal tube (same diameter) affect measurements?

Please see previous comment.

8. The tables and figures should probably be of higher quality.  
The question was discussed with the editor and seems to be related to the quality of the pdf document created for the review process.

**Reviewer #2:**

*Summary:* This paper is a nice state of the art summary and protocol regarding respiratory measurements in mice. It's well written with proper technical details. I only have a few minor comments for clarification purposes or aiding readability.  
  
*Major Concerns:*  
No major issue.  
  
  
*Minor Concerns:*  
1) The Introduction is very good. It serves the purpose. A minor point is on line 10: Most reader may not know about the technical details why a set of mutually prime frequency are advantageous over the integer multiple frequencies. Thus, I'd either add a reference or just say that it is possible to use multifrequency input signal together with Fourier analysis to obtain impedance simultaneously over a range of frequencies.

The authors re-worded the part of the introduction on multi-frequency signals in order to take the reviewers comment in consideration and to provide readers with additional references should they wish to learn more on the subject.

2) Table 1: paralytic agents are not always used and often not recommended. Also, the quality of Tables and figures is not good.

The heading in table 1 was modified. The regimens presented show a range of anesthetic regimens with or without paralytic agents.

The quality of the tables and figures was discussed with the editor. It seems to be related to the quality of the pdf document created for the review process.

3) Preparatory steps 2.3: Typo "Connect to the animal"

The text was corrected.

4) Since the entire procedure is based on the Flexivent, it would make sense to also include the actual script for the experiment.

Since many scripts were used to generate all the results presented in the paper, the authors choose to present a predefined script to measure respiratory mechanics.

5) The Results section often refers to the data as "lung mechanics"; however, what you get is respiratory system mechanics. It is true that in the mouse respiratory mechanics is mostly lung mechanics due to the very floppy nature of the chest wall, but you should point this out otherwise the procedure will confuse the naïve reader that he/she will be getting directly lung mechanics parameters.

The text was corrected.

6) Results, 2nd para: The statement "a parameter closely related to tissue resistance that is dominated by the resistance of the small airways and lung tissues" is confusing. G is a parameter representing tissue viscoelasticity, in this case, lung and chest wall tissues, although the latter contributes little except perhaps at very high lung volumes. Small airway resistance is absorbed in the parameter Rn. In the case of heterogeneous airway constriction, part of the airway flow resistance is folded into the parameter G making it sensitive to heterogeneities.

The text was modified. A reference to a recent paper (Siddiqui et al. 2012, PLoS ONE 7, e29381) which suggests that G is sampling mechanical changes from the peripheral airways was added to the text.

7) Discussion, 2nd para: I believe it is also extremely important that lung volume history is standardized otherwise recruitment-derecruitment issues can mess up the data and the results easily become ambiguous.

Standardization of lung volume was added as a critical step in the procedure as well as in the discussion.

8) Discussion 3rd para: Statement "the applied oscillatory waveforms applied to the lung are either too small or diverted, which results in datasets being excluded by the system". It depends on whether the system is a pressure or volume generator or something in between. If it is a volume generator, then the pressures increase depending on the input load impedance of the mouse. It is unclear what you mean by "diverted". As an alternative solution when the fit is bad, one can use more advanced mathematical models that incorporate heterogeneities. This is a limitation of the current protocol that should added to the discussion.

The sentence was re-worded in order to simplify the text. The suggested alternative was mentioned and a reference was added. In the previous statement, the authors had used the word “diverted” to refer to shunting of the oscillatory flow.