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Abstract:	<p>The forced oscillation technique (FOT) is a powerful, integrative and translational tool permitting the experimental assessment of lung function in mice in a comprehensive, detailed, precise and reproducible manner. It provides measurements of respiratory system mechanics through the analysis of pressure and volume signals acquired in reaction to predefined, small amplitude, oscillatory airflow waveforms, which are typically applied at the subject's airway opening. The present protocol details the steps required to adequately execute forced oscillation measurements in mice using a computer-controlled piston ventilator (flexiVent; SCIREQ Inc, Montreal, Qc, Canada). The description is divided into four parts: preparatory steps, mechanical ventilation, lung function measurements, and data analysis. It also includes details of how to assess airway responsiveness to inhaled methacholine in anesthetized mice, a common application of this technique which also extends to other outcomes and various lung pathologies. Measurements obtained in naïve mice as well as from an oxidative-stress driven model of airway damage are presented to illustrate how this tool can contribute to a better characterization and understanding of studied physiological changes or disease models as well as to applications in new research areas.</p>
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RE: Submission of revision to JoVE (JoVE50172)

Dear Dr Messmer-Blust,

My co-authors and I would like to thank you and the reviewers for their feedback on our manuscript. We addressed all the comments expressed in the attached reply as well as in the text.

We look forward to your reply,.

Sincerely,

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Evaluation of respiratory system mechanics in mice using the forced oscillation technique

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Keywords: forced oscillation technique, respiratory system mechanics, airway hyperresponsiveness, *flexiVent*, asthma, lung physiology, oxidative stress.

SHORT ABSTRACT

The present protocol provides a detailed step-by-step description of the procedures required to execute measurements of respiratory system mechanics as well as the assessment of airway responsiveness to inhaled methacholine in mice using the forced oscillation technique (*flexiVent*; SCIREQ Inc, Montreal, Qc, Canada).

LONG ABSTRACT

The forced oscillation technique (FOT) is a powerful, integrative and translational tool permitting the experimental assessment of lung function in mice in a comprehensive, detailed, precise and reproducible manner. It provides measurements of respiratory system mechanics through the analysis of pressure and volume signals acquired in reaction to predefined, small amplitude, oscillatory airflow waveforms, which are typically applied at the subject's airway opening. The present protocol details the steps required to adequately execute forced oscillation measurements in mice using a computer-controlled piston ventilator (*flexiVent*; SCIREQ Inc, Montreal, Qc, Canada). The description is divided into four parts: preparatory steps, mechanical ventilation, lung function measurements, and data analysis. It also includes details of how to assess airway responsiveness to inhaled methacholine in anesthetized mice, a common application of this technique which also extends to other outcomes and various lung pathologies. Measurements obtained in naïve mice as well as from an oxidative-stress driven model of airway damage are presented to illustrate how this tool can contribute to a better characterization and understanding of studied physiological changes or disease models as well as to applications in new research areas.

INTRODUCTION

Adequate characterization of the mechanical properties of the lungs in small animals has become essential since the burgeoning of murine models in respiratory science. When performed using the forced oscillation technique (FOT), a technique also used in human subjects, these measurements provide a powerful, integrative and translational approach to study meaningful physiological changes. FOT measurements are typically obtained by analyzing pressure and volume signals acquired in reaction to a predefined, small amplitude, oscillatory airflow waveform (also referred to as perturbation or input signal) applied at the subject's airway opening¹. In its simplest form, a FOT perturbation would be a single sinusoidal waveform at a well-defined frequency. More complex perturbations typically consist of a superposition of a selection of specific (mutually prime) frequency waveforms covering a broad spectrum. The decomposition of the multi-frequency input and output signals into their constituents using the Fourier transform allows the calculation of respiratory system input impedance (Z_{rs}), i.e. the transfer function between the input and output signals, at every frequency included in the perturbation². Therefore, FOT permits the simultaneous assessment of respiratory mechanics over a range of frequencies in a single maneuver². Fitting advanced mathematical models (e.g. the Constant Phase Model³) to the impedance data then permit a partitioning of the response into airway (central and peripheral) and parenchymal lung tissue dependent parameters^{1, 3}. Because many factors influencing the physiological response (e.g. breathing frequency, tidal volume, lung volume, upper airways, spontaneous breathing efforts, timing of measurements) are controlled and standardized by the measurement system and experimental procedures,¹ the technique is capable of generating precise and reproducible measurements provided that it is performed correctly. The objective of this article is to provide a detailed, chronological description of the procedure needed in order to execute such measurements in mice. The protocol consists of four parts: preparatory steps (reagents, equipment and subjects), mechanical ventilation, lung function measurements, and data analysis. Examples of representative results of respiratory system mechanics generated using a computer-controlled piston ventilator (*flexiVent*, SCIREQ Inc, Montreal, Qc, Canada) are provided. These were obtained from naïve mice as well as from an oxidative-stress driven model of airway damage characterized by airway inflammation, epithelial cell damage and increased airway responsiveness to inhaled aerosolized methacholine⁴. While this protocol is often used to assess airway responsiveness to inhaled methacholine, it extends to other outcomes and various pathologies including asthma, chronic obstructive pulmonary disease (COPD), emphysema, lung fibrosis, lung injury as well as to transgenic mouse models of pathologies similar to human disease. Research findings using this tool can contribute to a better characterization and understanding of physiological changes or disease models as well as to expansion into new research areas.

PROCEDURE

The procedures described below were approved by McGill University Institutional Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

1. Preparatory steps

1.1. Solutions:

- 1.1.1. Methacholine: Prepare a stock solution at 50 mg/ml and make serial dilutions (1:1) based on the concentrations to be tested⁵. Allow the solutions to reach room temperature before nebulizing⁵.
- 1.1.2. Anesthetic agents: Different regimens have been reported in the literature in various strains of mice (Table 1). **Note:** Regimen 1 was used under the present protocol.

1.2. Equipment: The present protocol applies to either of the two *flexiVent* generations supported by *flexiWare 7* software. The software functions are grouped under three modules: *Study Definition & Planning*, *Experimentation Session* and *Review & Reporting*.

- 1.2.1. Turn on the system (*flexiVent FX* only) and/or start the software.
- 1.2.2. At the first experimentation session or any time before it, open the *Study Definition & Planning* module to predefine the study structure.
- 1.2.3. Click on the *Create a new study* button and follow the wizard to create a study, outline the protocol and define the experimental groups and subjects to be studied.
- 1.2.4. Initiate an experimentation session by opening the *Experimentation Session* module and following the start-up sequence for study and template selection.
- 1.2.5. Assign a subject to the measurement site and confirm its weight.
- 1.2.6. Proceed with the calibration of the system by following the steps described in the operating software. You will be prompted at one point to attach the cannula to be used (step 1.3.3) to the Y-tubing for calibration.
- 1.2.7. **Critical step.** Repeat step 1.2.6 if the calibration values obtained are outside the acceptable range. (Refer to the *flexiVent FX* or *flexiWare 7* user manual for the module specific acceptable ranges of calibration values).
- 1.2.8. Cancel prompts to start ventilation and data recording unless ready to start the experiment. These can be initiated at a later point.

1.3. Subjects:

- 1.3.1. Anesthetize the subject using appropriate doses of anesthetic agents (Table 1).
- 1.3.2. Verify that the subject has reached a surgical level of anesthesia. The subject should show no reaction to a toe pinch and its breathing should be regular and not laboured.
- 1.3.3. Perform a tracheotomy and cannulate the trachea.
 - 1.3.3.1. Place the animal on its back and provide a source of heat (e.g. temperature controlled heating blanket or a lamp with a 60 watt bulb situated approximately 45 cm from the mouse to avoid excessive heating).

- 1.3.3.2. Clean the throat area with alcohol and expose the trachea by making a skin incision and gently separating the submaxillary gland and the muscle layer covering it.
- 1.3.3.3. Gently lift the trachea using a pair of micro-forceps, pass a suture underneath it and tie a loose knot on top of the trachea.
- 1.3.3.4. Cut between two rings of cartilage nearest the larynx to make a small incision in the trachea without sectioning it.
- 1.3.3.5. Insert the previously calibrated cannula into the incision and advance it gently inside the trachea the length of approximately 5 rings. **Note:** The present experiments were conducted using a 1.2 cm long metal 18 gauge cannula.
- 1.3.3.6. **Critical step.** Secure the cannula in place using the suture. The attachment should form an airtight seal around the cannula.

2. Mechanical ventilation

- 2.1. Bring the animal close to the ventilator.
 - 2.2. Start mechanical ventilation by selecting a predefined or a customized ventilation profile in the ventilation docker.
 - 2.3. Connect the animal to the ventilator via the Y-tubing.
 - 2.4. **Critical step.** Align the animal to the ventilator and ensure that the tracheal cannula is at the same level as the ventilator to avoid a possible cannula occlusion or tracheal twist.
 - 2.5. **Critical Step.** Execute a *Deep Inflation* perturbation by double-clicking on the perturbation name to verify the cannula insertion and attachment. In absence of a leak, the system should be able to hold a pressure of 30 cmH₂O over a 3 second period without excessive volume displacement (Figure 1). The recorded volume and pressure traces should also be smooth with no signs of offset or deformation as these could indicate a cannula obstruction or misplacement.
 - 2.6. If needed, connect vital sign transducers for heart rate and body temperature monitoring. Data recording can be initiated either manually or automatically via a script.
3. **Lung function measurements.** Measurements or commands (e.g. nebulizer activation, event markers) can be automated using predefined or customized scripts for a highly controlled and repeatable experimental process (Figure 2). Six families of perturbations giving rise to a number of parameters can be used to describe the subject respiratory system mechanics at baseline and following a given challenge (Table 2).
- 3.1. **Critical step.** When ready to start taking measurements, run a *Deep Inflation* to recruit closed lung areas and standardize lung volume history.
 - 3.2. **Critical step.** Verify the absence of spontaneous inspiratory efforts by running a test measurement (e.g. PVs-P or PVs-V). Observe the pressure signal traces in the *Selected Dataset* view. With stepwise PV curves, pressure plateaus should be well defined with no

downwards deflections. A downward swing in pressure would indicate an inspiratory effort from the animal (Figure 3).

3.3. Initiate the selected script by double-clicking on its title. Scripts used in the present study generally included for measurements:

- A sequence of baseline measurements in triplicate.
- Activation of the nebulizer for assessment of airway responsiveness to inhaled methacholine. **Note:** When prompted by the system, load approximately 100 µl of saline or of a solution of methacholine into the nebulizer. Nebulization will start and stop automatically.
- A sequence of closely spaced measurements (every 10-15 s) for a period of approximately 3 minutes following the activation of the nebulizer.
- A prompt to perform another challenge and repeat a sequence of measurements. **Note:** Drying the inside the nebulizer mount with a swab in between challenges can help prevent droplets or condensation from building-up in the inspiratory line.

3.4. At the end of the experiment, stop ventilation and detach the subject.

3.5. Switch to the next subject in the operating software and confirm its weight.

3.6. **Critical step.** Rinse and dry nebulizer, Y-tubing, and cannula between subjects.

3.7. Repeat steps 1.2.6 to 3.6.

3.8. At the end of the day, close the experimental session. Remember to rinse and dry nebulizer, Y-tubing, and cannula and to clean the system expiratory valve before leaving the laboratory by following the manufacturer's instructions.

4. Data Analysis. The software automatically calculates and displays parameters associated with a perturbation. It also provides a coefficient of determination (COD) which reflects the fit of the mathematical model to the data. Each dataset with an insufficient COD is labelled as excluded by the software. Review of experimental sessions, data re-analysis and creation of export scenarios are done in the *Review & Reporting* module of the software.

4.1. Open the *Review & Reporting* module and create an export scenario, taking care to only include datasets having a sufficient COD.

4.2. Export as needed parameters, pressure- or flow-volume curve, raw dataset signals or subject information to a spreadsheet application (see Table 3).

4.3. Average baseline measurements for each parameter and plot all measurements as a function of time (see Figure 4). You may then choose to calculate the area under the curve, analyze the general profile of the curves or perform a statistical analysis.

4.4. To express airway responsiveness results as a function of the concentration of methacholine, determine for each subject, parameter and experimental condition either a specific point (e.g. peak) or a specific time after each methacholine challenge. Calculate group averages and report or plot results for each experimental condition (Table 4, Figure 5).

4.5. You may also consider calculating the concentration producing a doubling of a given parameter baseline value (PC₂₀₀; Figure 5C), applying a normalization (e.g. % baseline) or performing a statistical analysis.

REPRESENTATIVE RESULTS

Respiratory system mechanics measurements. Table 4 shows typical results from naïve A/J mice obtained at baseline and following methacholine-induced bronchoconstriction (12.5 mg/ml) using either of the two *flexiVent* generations supported by *flexiWare 7* software. Mechanics of the respiratory system, i.e. under closed chest conditions, were assessed by alternating perturbations of the single and broadband frequency forced oscillation families in a closely spaced manner (SnapShot-150, Quick Prime-3, respectively). Since ventilation is paused during measurements, the Quick Prime-3, which covers a similar frequency range as the Prime-8 but has a shorter duration (3 vs 8 seconds), was selected in order to shorten the apneic period, minimize the effect of the perturbation on blood gases and provide a better resolution of the response. Parameters associated with each perturbation were calculated automatically by the operating software. Results illustrate that the two generations of the *flexiVent* system produced equivalent measurements of respiratory mechanics.

Site of lung response. Distinguishing the site of lung response allows the investigator to further pinpoint affected regions as well as to identify potential points of pharmacological intervention⁶. For example, naïve A/J mice show an increase of baseline resistance when the end expiratory pressure against which measurements are made is increased from 3 to 9 cmH₂O (Figure 6A, SnapShot-150). In the present example, the use of broadband FOT measurements (Quick Prime-3) provided details to clarify the basis for the change in resistance: The change in end expiratory pressure resulted in a decrease in the airway resistance (R_N) consistent with the bronchodilating effects of a higher lung volume and the larger inflation pressure (Figure 6D) and an increase in tissue damping (G; Figure 6E), a parameter closely related to tissue resistance that reflects tissue viscoelasticity and possibly the resistance of the small airways⁷. The latter is known to increase with increasing lung volume.

Airway hyperresponsiveness. Following chlorine gas exposure, airway responsiveness to inhaled methacholine increases compared to air exposure in Balb/c mice as a result of airway damage⁴ (Figure 2). Chlorine is known to induce oxidative stress, leading to destruction of structural cells in the airways, in particular epithelial cells, and inducing recruitment of inflammatory cells. As represented in Figure 5, changes in all parameters describing respiratory system mechanics can be seen in response to increasing methacholine challenges. In comparison to air-exposed mice, mice exposed to chlorine gas displayed greater maximal responses at all FOT parameters (Figure 5A, B, D-F) as well as a statistically significant leftward shift of the concentration-response curve exemplified by a reduction of the concentration of methacholine required to cause a doubling in resistance and elastance (PC₂₀₀; Figure 5C). Those results illustrate, respectively, airway hyperresponsiveness and hypersensitivity to inhaled methacholine following exposure to chlorine gas.

Other measurements. In addition to FOT, the *flexiVent* system can also be used to collect other types of lung function⁸⁻¹⁰ or cardiovascular¹¹ measurements. Figure 7 displays a representative stepwise, pressure-driven pressure-volume curve in naïve A/J mice under baseline conditions. The upper portion of the deflation limb of the curve is fit to the Salazar-Knowles equation¹² and parameters are automatically calculated by the software.

DISCUSSION

The continued study of airway dysfunction as it relates to asthma and other lung disorders remains paramount to the understanding of underlying mechanisms of disease and the development of treatment options. The use of mice to model airway disease has been essential in gaining understanding into these disease mechanisms. When considering evaluating airway dysfunction in a subject as small as a mouse, having reliable and accurate tools by which to measure lung function is critical. Furthermore, having tools capable of providing insights on the location of airway dysfunction or therapeutic effect is invaluable. The FOT technique combines all these attributes and provides a powerful, integrative and translational approach to evaluate physiological changes.

In order to succeed with this type of measurement in mice, special attention should be given to a few steps, namely the calibration of the system, the resistance of the endotracheal cannula, the type of nebulizer (as well as its operating settings) the positioning of the animal and the standardization of lung volume history. In addition, it is imperative in order to obtain valid datasets that the subject's respiratory system remains passive during measurements. This can be achieved by the administration of a muscle paralyzing agent, working at a deep plane of anesthesia or by hyperventilating the subject to induce apnea (see Table 1). Investigators can start by mastering the system and its operating software, if desired, with test loads. The natural next steps would then be to acquire the necessary skills for measurements in mice and to generate reproducible results in naïve animals before moving to disease models or treated mice. Since an important proportion of disease models in respiratory research involve exposing animals to agents such as allergens, toxins, pollutants, cigarette smoke or gases, variability in the results obtained with the measurement technique described in this article could therefore be influenced by the exposure procedure used. Standardization of key experimental processes (e.g. using computer-controlled exposure and measurement systems^{6, 13, 14}) could potentially have a significant impact in reducing variability.

Examples presented in this article represent a selection of typical results from naïve and chlorine-exposed mice experiments while highlighting the strengths as well as the limitations of the technique. As can be seen for example in Figure 6, the technique is capable of generating reproducible lung function measurements. While similar baseline resistance values were reported between mouse strains, differences in elastance were however observed¹⁵. Substantial changes are also to be expected between infant and adult mice¹⁶. As for other *in vivo* physiological assessment, high precision results, such as those generated by the FOT, come with a concession as to the natural state of the subjects. This principle, which is referred to as the phenotyping uncertainty principle¹, applies to the present protocol in the sense that measurements need to be made in anesthetized, tracheotomised (or orally intubated) and mechanically ventilated subjects. Another limitation of the technique is observable in figure 5 (D-F) where data are not available at the highest concentrations for the chlorine-exposed group because the fit of the Constant Phase Model to the data is poor above moderate levels of bronchoconstriction. However, severely bronchoconstricted animals could be assessed by analyzing Zrs directly¹⁵, or by using third party post-analysis software to fit more complex mathematical models, e.g. taking into account the heterogeneity of mechanical function¹⁷. Excluded datasets can also be observed if the animal's airways are not sufficiently passive or if the resistance of the cannula is too high. As a rule of thumb, the resistance of the cannula should not exceed the animal's resistance at baseline.

Working with a cannula of larger inner diameter and/or shorter length will help reduce the cannula resistance. Finally, the present demonstration of FOT measurements in mice may be perceived as a time-consuming and therefore less efficient methodology or less applicable to longitudinal studies compared to less invasive techniques. However the latter are associated with a great deal of uncertainty as to the basis of their outcomes and are view by many as being flawed¹. Repeated invasive measurements are possible in orally intubated animals, although technically more challenging¹⁷.

From the examples provided, results demonstrated the equivalence of the two generations of the *flexiVent* system at producing measurements of respiratory mechanics, as well as airway hyperreactivity and hypersensitivity to inhaled methacholine following chlorine-exposure in mice. When used to characterize or understand physiological changes or disease models, the detailed measurement aspect related to the technique can contribute to extend the current state of knowledge.

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DISCLOSURE

AR, LF, TFS are employed by SCIREQ Scientific Respiratory Equipment Inc. TFS also owns stock.

AUTHORS' CONTRIBUTION

All authors took part in the conception of the manuscript. In addition, TKMcG initiated the project, collected experimental results, contributed to the writing of the manuscript and its critical review. AR collected and analyzed experimental results, drafted the manuscript and contributed to its critical review. LF collected experimental results and contributed to the critical review of the manuscript. TFS and JGM contributed to the critical review of the manuscript.

TABLES

Table 1: Examples of anesthetic regimens used in mice.

Table 2: Perturbations used for lung function measurements in mice.

*Extension required to the system. The subject also needs to be in a closed plethysmograph chamber during measurements.

Table 3: Example of exported parameters from the single and broadband frequency forced oscillation perturbation families.

Table 4: System Comparison.

Comparison of lung mechanics parameters collected using the two generations of the *flexiVent* system operated by *flexiWare 7* software. Results were generated in naïve A/J mice (n=5/group) at baseline and following methacholine-induced bronchoconstriction (Mch 12.5 mg/ml).

*Groups were compared using a two-way ANOVA for repeated measurements and the \log_{10} of individual responses for homogeneity of variances (GraphPad Prism, version 5.03; GraphPad Software, San Diego, USA).

FIGURE LEGENDS

Figure 1: Screenshot of a deep lung inflation. The upper panel shows the volume displaced by the piston of the ventilator (red trace) and the volume delivered to the subject (grey trace). The lower panel displays the cylinder pressure increasing to a set pressure of 30 cmH₂O over a period of 3 seconds and held constant for the same time period.

Figure 2: Example of a typical script used to assess respiratory system mechanics at baseline

Figure 3: Spontaneous inspiratory efforts during the execution of a stepwise pressure-volume curve.

Figure 4: Time-course response following increasing inhaled methacholine challenges. Results are expressed as the mean (\pm standard deviation) of a group of 5 naïve spontaneously hyperresponsive A/J mice.

Figure 5: Changes in respiratory system mechanics following increasing methacholine challenges in chlorine- and air-exposed Balb/c mice. Peak value was identified for each parameter in every subject and experimental condition. Group averages were then calculated (mean \pm standard deviation; n=4-6). Differences between groups were assessed by analysis of variance using the log₁₀ of individual responses for homogeneity of variances. The concentration of methacholine producing a doubling of baseline (PC₂₀₀) was obtained by fitting a second order polynomial to individual dose-response curves and interpolation of the fitted curve. Data points are missing in figure 2D, E and F in the chlorine-exposed mice at the two highest methacholine concentrations due to insufficiently high coefficients of determination reflecting a poor fit of the mathematical model to the data.

Figure 6: Partitioning of the respiratory response into airway and lung tissue mechanics. Experimental trace from a naïve A/J mice illustrating single (2.5 Hz) and broadband (1-20.5Hz) frequency forced oscillation measurements of respiratory mechanics in triplicate at two different end expiratory pressures (3 & 9 cmH₂O).

Figure 7: Pressure-volume curve in naïve A/J mice under baseline conditions. Pressure-volume curves were generated using a stepwise pressure-driven perturbation (PVs-P) to ensure that each mouse lungs were inflated to the same pressure, independently of their condition. Salazar-Knowles equation parameters extracted from individual pressure-volume curves were also averaged and reported in a table format. Results are expressed as the mean \pm standard deviation (n=6).

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Table 1
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Regimen	Pre-anesthetic medication (mg/kg, ip)	Anesthetic Agents (mg/kg, ip)	Support Treatment	References
1	<ul style="list-style-type: none">• Xylazine (8-12)• Ketamine (130) / Diazepam (7)	Sodium Pentobarbital (30-70)	Pancuronium (0.8 - 1.2 mg/kg, ip)	4, 19-21
2		Sodium Pentobarbital (90)	± Pancuronium (0.8 µg/kg, ip)	22, 23
3		Xylazine (2-18) / Ketamine (40-65)	± hyperventilation	6, 24, 25
4		Diazepam (17.5) / Ketamine (450)		26
5		Avertin (240)	Pancuronium (0.03 mg/kg, iv)	27
6		isoflurane (3%)	Pancuronium (1 µg, ip)	28

Table 2

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Perturbation Families	Perturbation Names	Typical Parameters
Recruitment maneuver	Deep Inflation	<ul style="list-style-type: none"> • IC: Inspiratory capacity
Single frequency forced oscillation	SnapShot-150	Single Compartment Model parameters: <ul style="list-style-type: none"> • R: resistance of respiratory system • E: elastance of respiratory system • C: Dynamic compliance
Broadband frequency forced oscillation	Quick Prime-3 Prime-8	Constant Phase Model parameters: <ul style="list-style-type: none"> • R_{N}: Newtonian resistance (resistance of the airways) • G: Tissue damping (related to tissue resistance) • H: Tissue elastance • η: ratio of G/H
Pressure - volume curve	PVs-V PVi-V PVs-P PVi-P	<ul style="list-style-type: none"> • Pressure-volume curve • C_{st}: Quasi-static compliance • Area: Area between the Inspiratory and expiratory limb of the pressure-volume curve • Selazar-Knowles parameter A: estimate of Inspiratory capacity • Selazar-Knowles parameter K: curvature of the upper portion of expiratory limb of P-V curve.
Negative pressure - driven forced expiration	NPFE*	<ul style="list-style-type: none"> • Flow-volume curve • FEV_{0.1}: forced expired volume in 0.1 s • FVC: forced vital capacity • PEF: peak expiratory flow
Gated Image acquisition		

Table 3
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		Single-Compartment Model				Constant-Phase Model			
Subject	Perturbation	R_m	C_m	E_m	COD	R_H	G	H	COD ₉₅
Mouse 2	SnapShot-150 v7.0	0.5978	0.0423	23.6534	0.9995				
Mouse 2	Quick Prime-3 v7.0					0.3381	4.0771	21.2276	0.9821
Mouse 2	SnapShot-150 v7.0	0.5935	0.0415	24.0712	0.9993				
Mouse 2	Quick Prime-3 v7.0					0.3286	4.1327	21.6388	0.9857
Mouse 2	SnapShot-150 v7.0	0.6005	0.0411	24.3310	0.9987				
Mouse 2	Quick Prime-3 v7.0					0.3334	4.0493	21.5056	0.9824

Table 4
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			<i>flexiVent</i> (1 st generation)		<i>flexiVent</i> FX		
Perturbation	Parameters (units)	Conditions Studied	Mean	SD	Mean	SD	p<0.05*
SnapShot-150	R (cm H ₂ O.s/ml)	Baseline	0.59	0.03	0.57	0.06	ns
		Mch	3.70	1.92	2.79	0.76	ns
	E (cm H ₂ O/ml)	Baseline	23.14	2.22	23.27	2.92	ns
		Mch	91.09	62.30	71.15	20.46	ns
Quick Prime-3	R _N (cm H ₂ O.s/ml)	Baseline	0.32	0.03	0.29	0.02	ns
		Mch	1.39	0.66	0.93	0.14	ns
	G (cm H ₂ O/ml)	Baseline	4.12	0.29	4.18	0.43	ns
		Mch	18.40	8.25	15.88	4.24	ns
	H (cm H ₂ O/ml)	Baseline	21.40	2.05	20.5	2.5	ns
		Mch	46.02	24.58	38.4	9.2	ns

Figure 1
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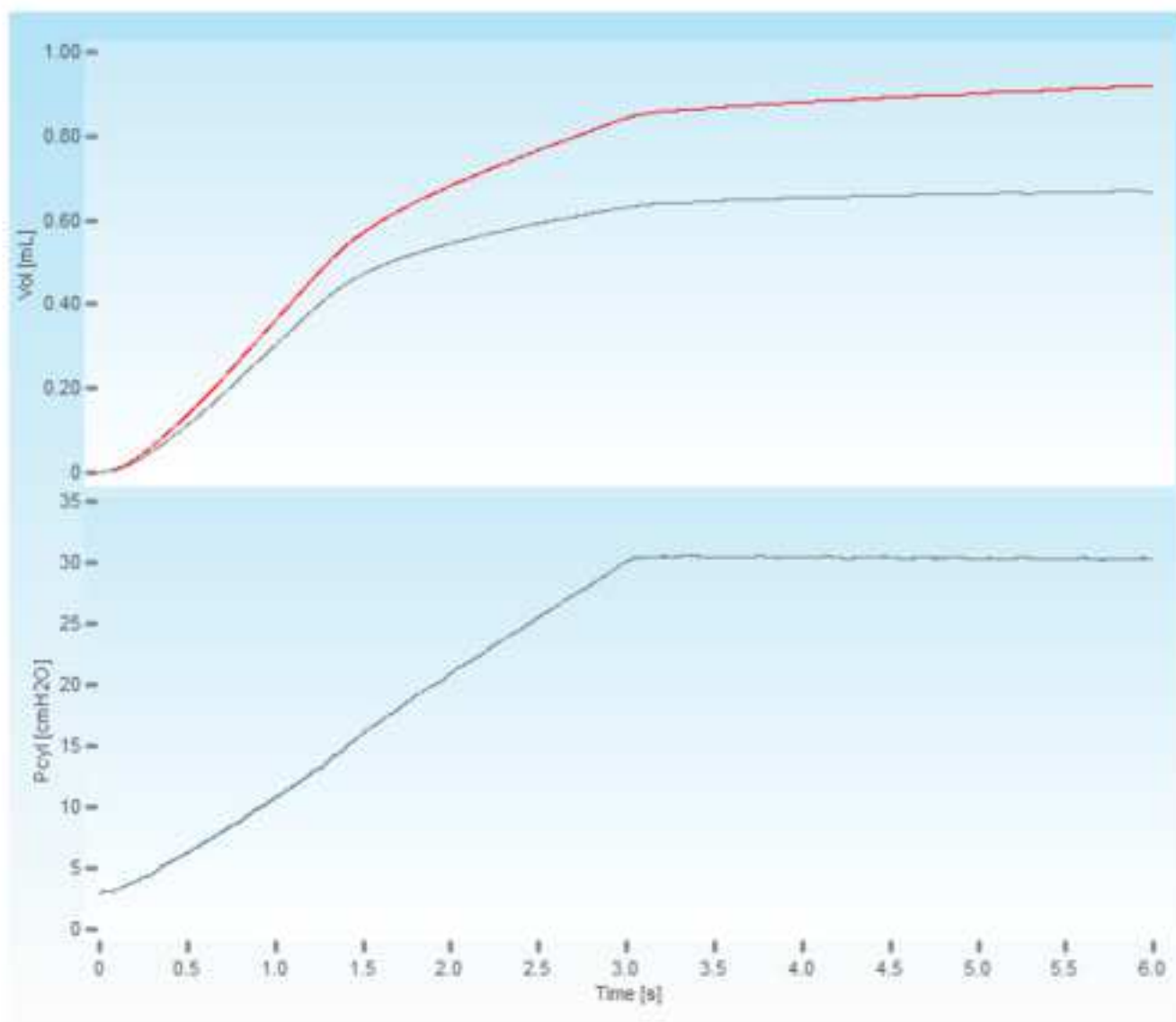


Figure 2

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StartScript

//start default ventilation

00:00 StartVentilation pattern='Mouse Default CMV'

//perform baseline measurements

00:00 loop count=3

00:28 Perturbation name='Deep Inflation v7.0'

00:12 Perturbation name='Snapshot-150 v7.0'

00:06 Perturbation name='Quick Prime-3 v7.0'

00:08 Perturbation name='PVs-P v7.0'

00:00 endloop

// useful shortcuts: CTRL-J for command templates

// CTRL-SPACE for completion

EndScript

Figure 3
[Click here to download high resolution image](#)

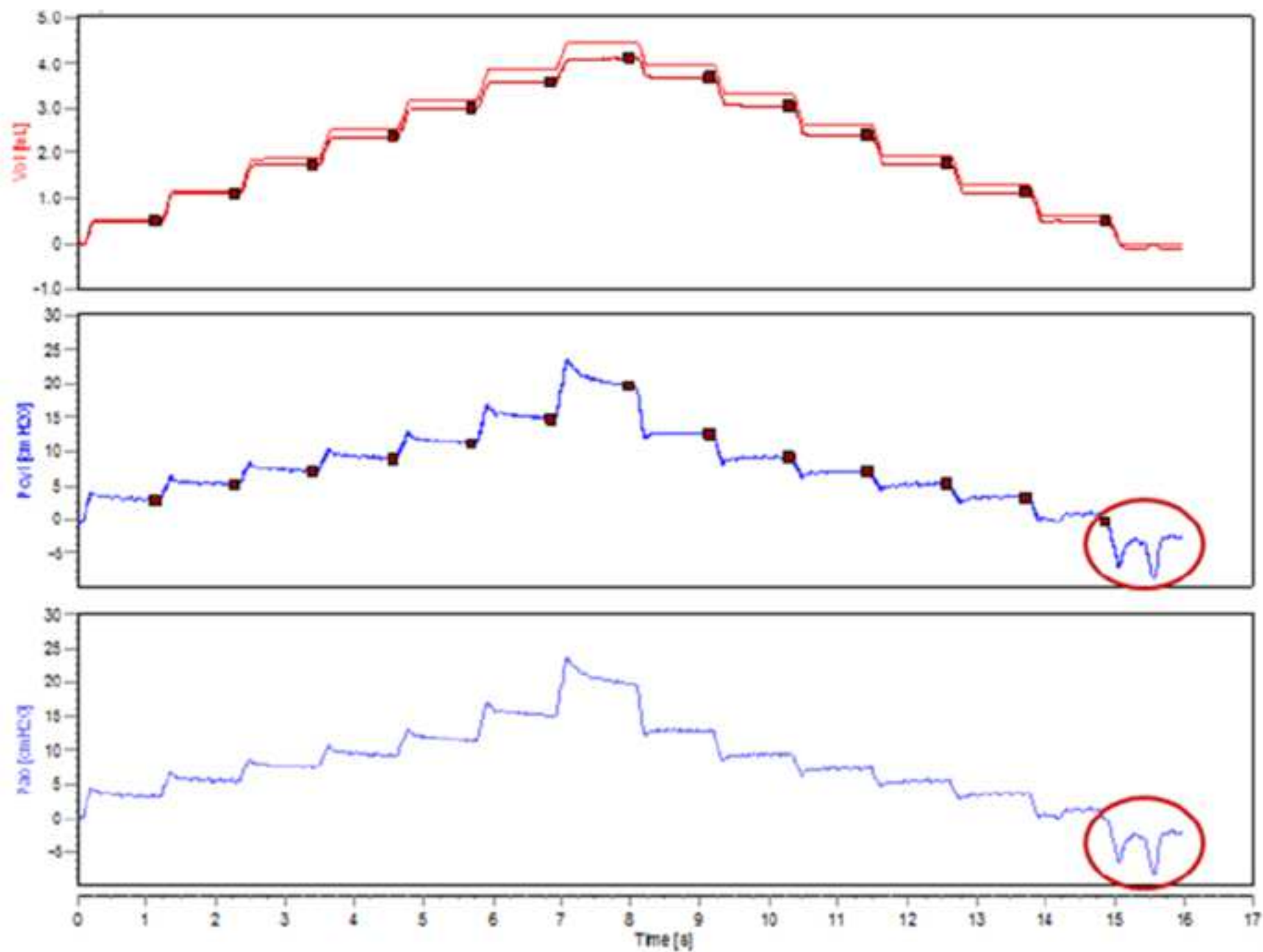


Figure 4

[Click here to download high resolution image](#)

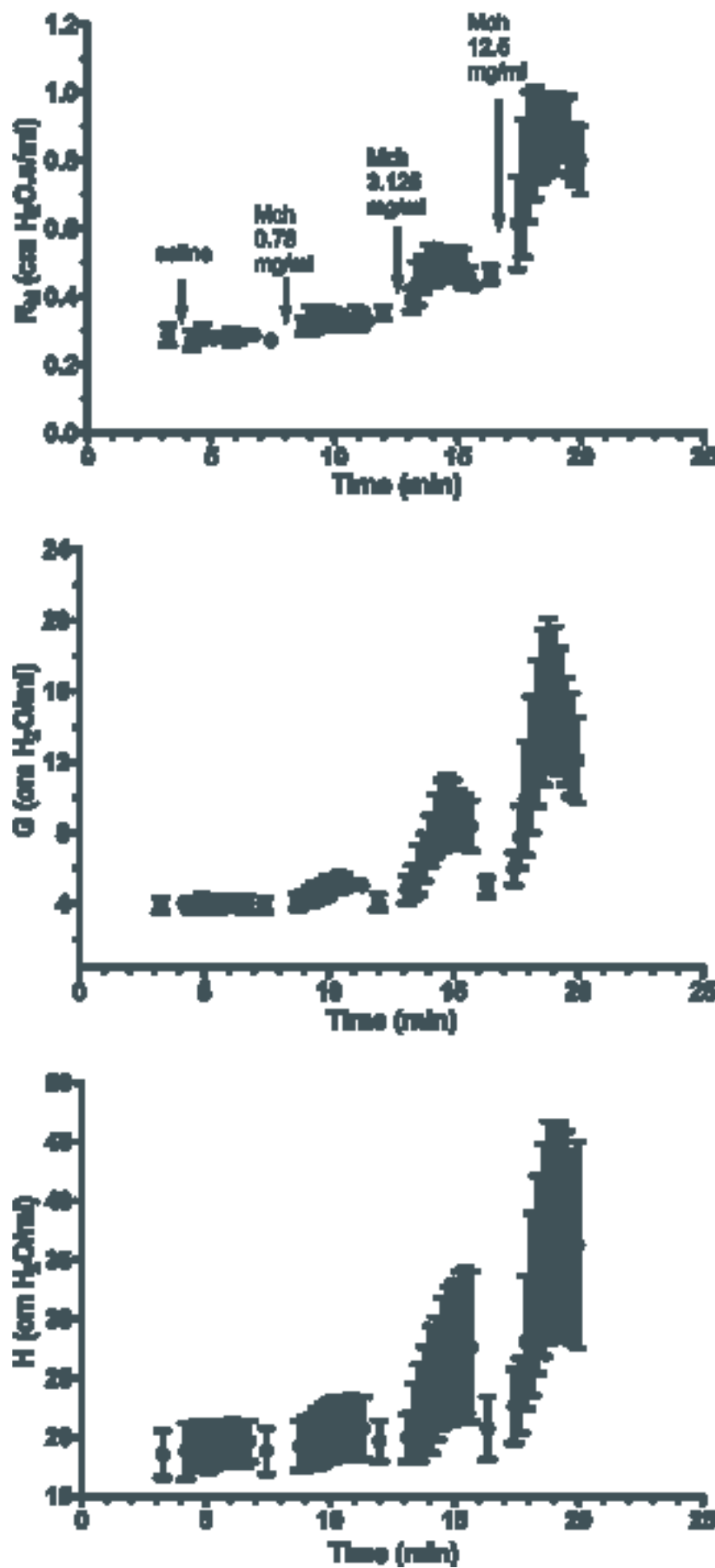


Figure 5
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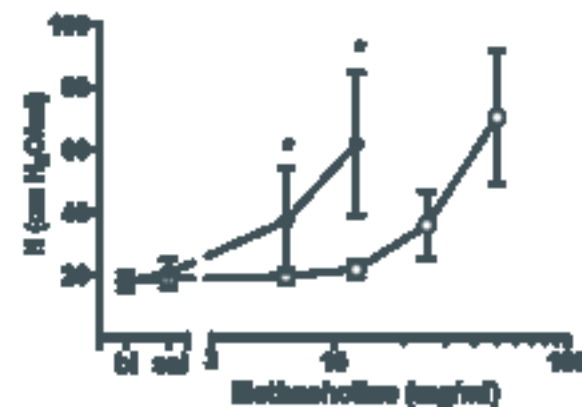
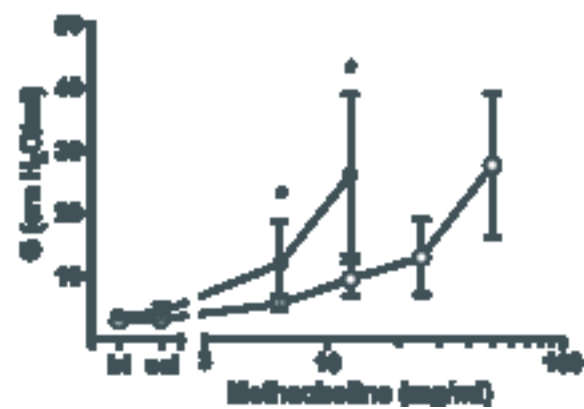
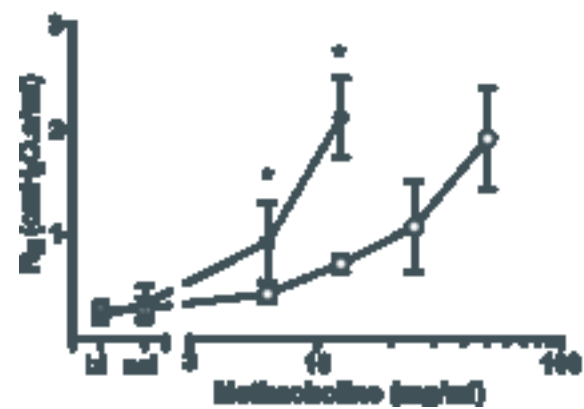
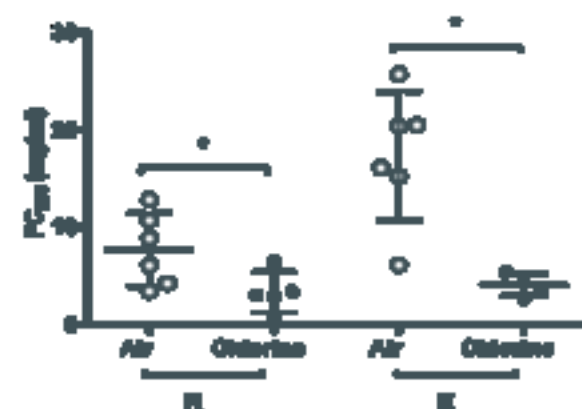
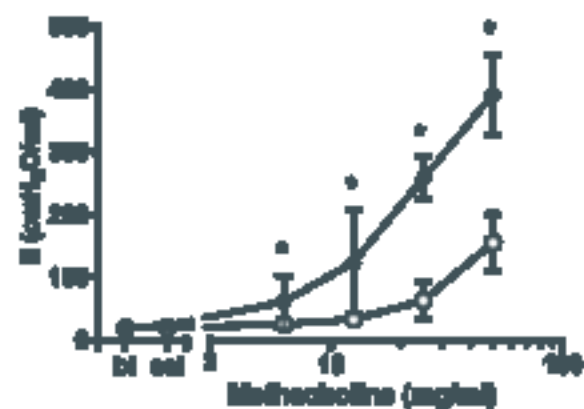
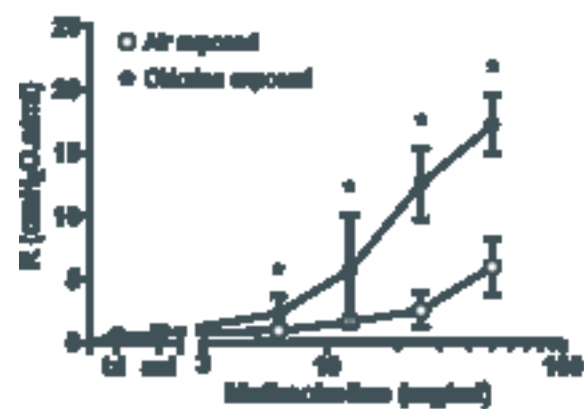


Figure 6
[Click here to download high resolution image](#)

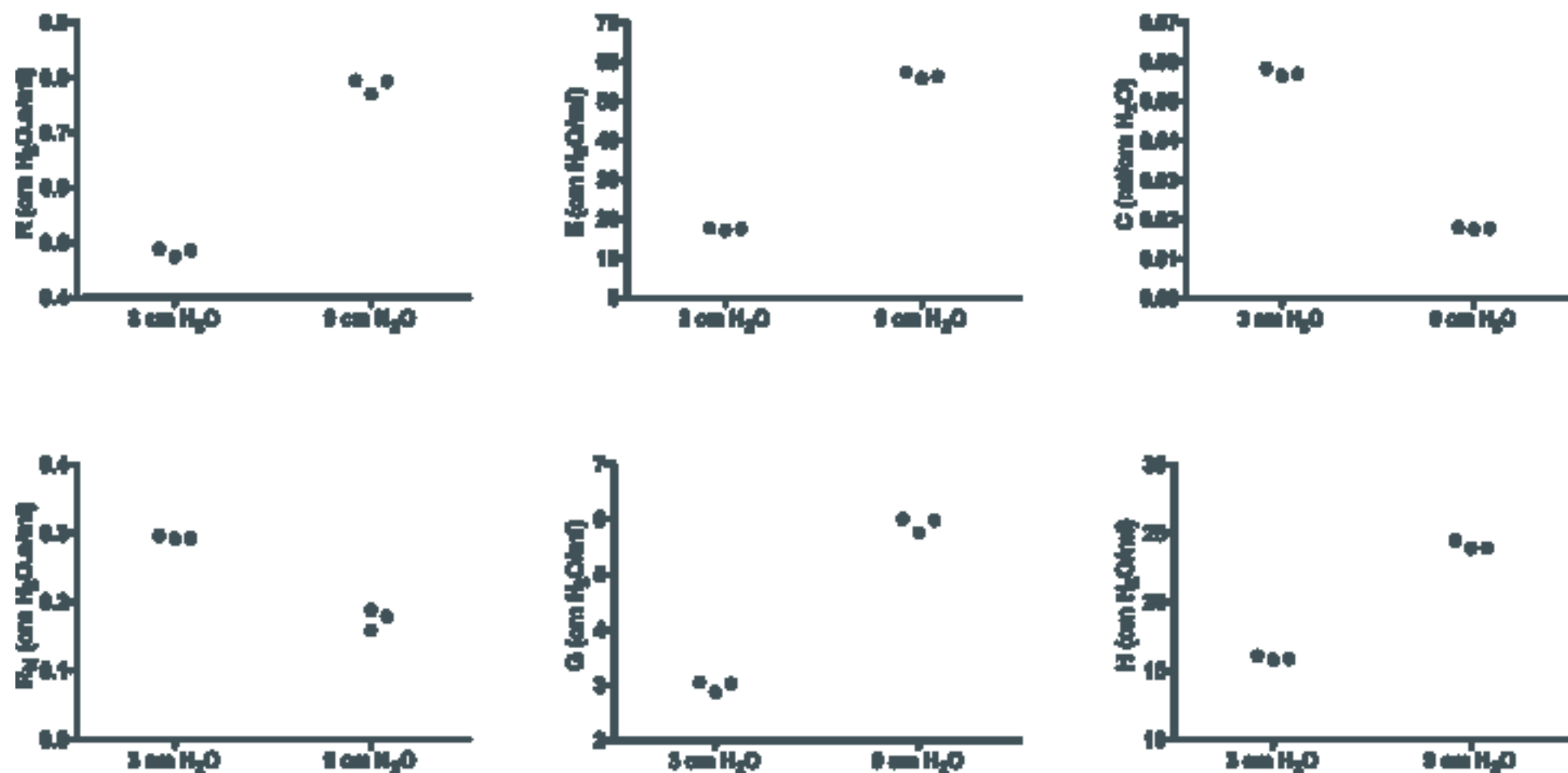
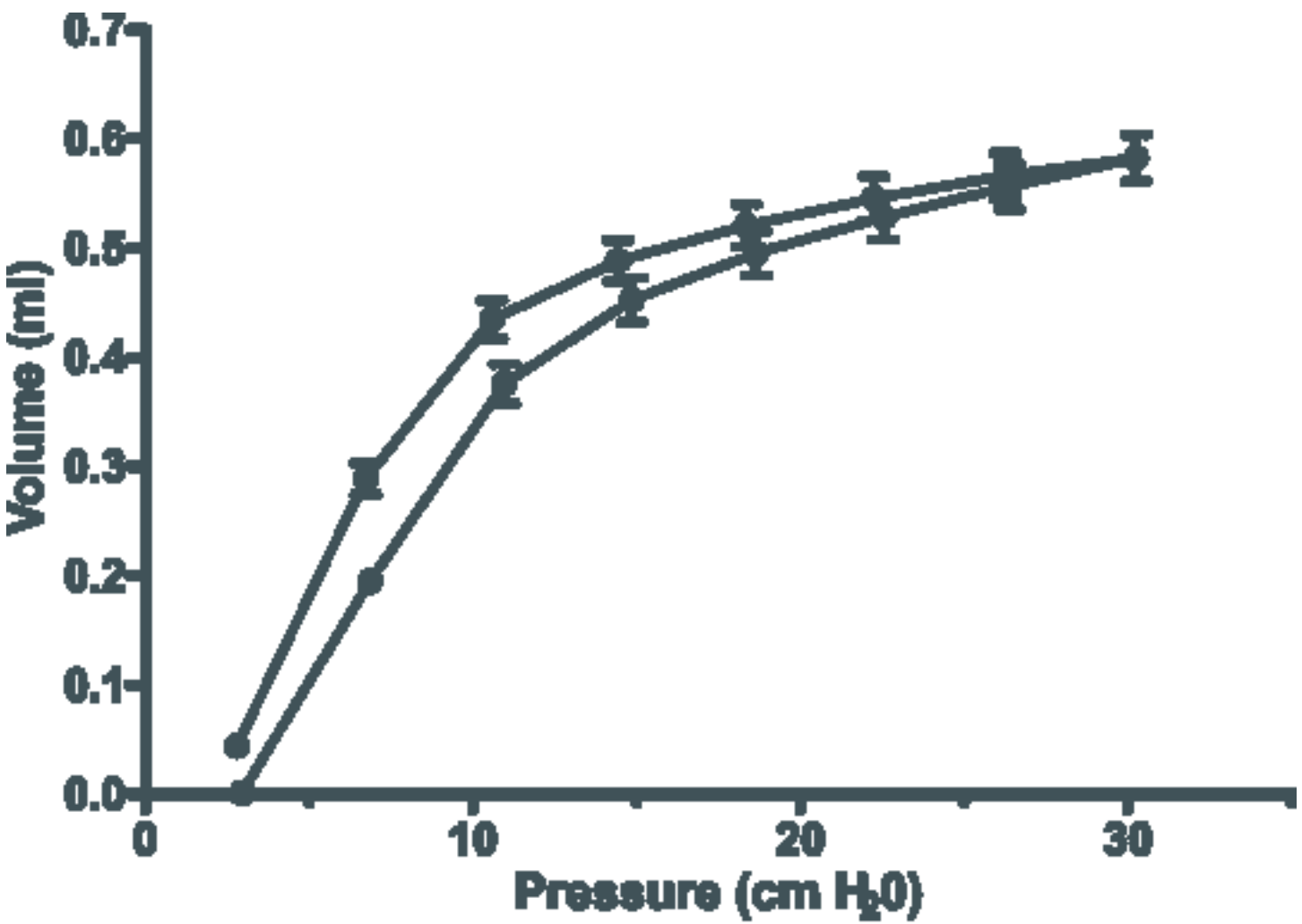


Figure 7
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Parameters	Mean	Standard Deviation	Coefficient of Variation (%)	n
A (ml)	0.564	0.041	7.20	6
K (/cmH ₂ O)	0.169	0.012	7.00	6
C _{st} (ml/cmH ₂ O)	0.06	0.007	11.20	6
Area (ml.cmH ₂ O)	1.174	0.094	8.00	6

REAGENTS

Name of Reagent/Material	Company	Catalogue Number	Comments
Acetyl-β-methylcholine chloride	Sigma-Aldrich	A-2251	Methacholine
Micro-Adson forceps, serrated 12 cm	Fine Science Tools	11018-12	
Moria MC31 forceps, serrated-curved	Fine Science Tools	11370-31	
Iris scissors-tough cut, straight 11.5 cm	Fine Science Tools	14058-11	
Spring scissors-2.5 mm blades, straight	Fine Science Tools	15000-08	
	Brico Medical		Endotracheal
Non-sterile blunt needle (18g x ½")	Supplies Inc.	BN1805	cannula
Non-sterile 5-0 silk suture	Seraflex	ID158000	
Phosphate buffered solution	Gibco	14190-144	
15 ml conical tubes	Starstedt	SS-4001	
1 ml TB syringes	Becton Dickinson	309626	
200 µl filter tips	Biosphere	70.760.211	

EQUIPMENT

Name of Equipment	Company	Catalogue Number	Comments
<i>flexiVent</i> FX	SCIREQ Inc.	sales@scireq.com	www.scireq.com
Aerogen Aeroneb nebulizer	SCIREQ Inc.	sales@scireq.com	www.scireq.com

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 be 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.