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TITLE:

Combining Volumetric Capnography and Barometric Plethysmography to Measure the Lung Structure-Function Relationship

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KEYWORDS

Lung volume, dead space, airways, capnography, plethysmography, pulmonary function, lung function

SUMMARY

Here, we describe two measures of pulmonary function – barometric plethysmography, which allows the measurement of lung volume, and volumetric capnography, a tool to measure the anatomic dead space and airways uniformity. These techniques may be used independently or combined to assess airways function at different lung volumes.

ABSTRACT

Tools to measure lung and airways volume are critical for pulmonary researchers interested in evaluating the impact of disease or novel therapies on the lung. Barometric plethysmography is

a classic technique to evaluate the lung volume with a long history of clinical use. Volumetric capnography utilizes the profile of exhaled carbon dioxide to determine the volume of the conducting airways, or dead space, and provides an index of airways homogeneity. These techniques may be used independently, or in combination to evaluate the dependence of airways volume and homogeneity on lung volume. This paper provides detailed technical instructions to replicate these techniques and our representative data demonstrates that the airways volume and homogeneity are highly correlated to lung volume. We also provide a macro for the analysis of capnographic data, which can be modified or adapted to fit different experimental designs. The advantage of these measures is that their advantages and limitations are supported by decades of experimental data, and they can be made repeatedly in the same subject without expensive imaging equipment or technically advanced analysis algorithms. These methods may be particularly useful for investigators interested in perturbations that change both the functional residual capacity of the lung and airways volume.

INTRODUCTION

Gas washout techniques have been used for decades to provide important information about the structure and uniformity of the airway tree. The lung is classically described as having two compartments – a conducting zone that is comprised of the anatomic dead space and the respiratory zone where gas exchange occurs in the alveoli. The conducting airways are termed as "dead space" because they do not participate in the exchange of oxygen and carbon dioxide. In the single breath gas washout method, the concentration profile of an exhaled gas can be used to determine the volume of the anatomic dead space and to derive information about the uniformity of ventilation. Some methods rely on the breathing of inert gases to make these measures (N_2, argon, He, SF_6, *etc.*). The use of inert gas is well-established, supported by scientific consensus statements[1], and there are available commercial equipment with user friendly interfaces. However, the exhaled profile of carbon dioxide (CO_2) can be used to derive similar information. Evaluating the profile of CO_2 as a function of the exhaled volume, or volumetric capnography, does not require the participant to breathe special gas mixtures and allows the investigator to gather additional information flexibly about metabolism and gas exchange with minimal adjustment to the technique.

During a controlled exhalation, the concentration of CO_2 can be plotted against the total exhaled volume. At the beginning of an exhalation, the dead space is filled with atmospheric gas. This is reflected in Phase I of the exhaled CO_2 profile where there is an undetectable amount of CO_2 (**Figure 1**, top). Phase II marks the transition to the alveolar gas, where gas exchange occurs and CO_2 is abundant. The volume at the midpoint of Phase II is the volume of the anatomic dead space (V_D). Phase III contains alveolar gas. Because airways with different diameters empty at different rates, the slope (S) of Phase III provides information about airways uniformity. A steeper slope of Phase III suggests a less uniform airway tree proximal to the terminal bronchioles, or convection-dependent inhomogeneity[2]. In the case where a perturbation may change the rate of CO_2 production, and to make comparisons between individuals, the slope can be divided by the area under the curve to normalize for differences in metabolism (NS or normalized slope). Volumetric capnography has been used previously to evaluate the changes in airways volume and uniformity following air pollutant exposure[3-6].

Gas transport in the lung is governed by both convection and diffusion. Single breath washout measures are highly dependent on air flow and the measured value of V_D occurs at the convection-diffusion boundary. Changing the flow rate of the exhalation or preceding inhalation changes the location of that boundary[7]. Capnography is also highly dependent on the volume of the lung immediately preceding the maneuver. Larger lung volumes distend the airways, resulting in larger values of V_D[8]. One solution is to consistently make the measurement at the same lung volume – usually functional residual capacity (FRC). An alternative, described here, is to couple volumetric capnography with barometric plethysmography, in order to obtain the relationship between V_D and lung volume. The participant then performs the maneuver at constant flow rates, while varying the lung volume. This still allows for classic capnographic measures to be made at FRC, but also for the relationship between the lung volume and dead space volume and between the lung volume and homogeneity to be derived. Indeed, the added value of coupling capnography with plethysmography comes from the ability to test hypotheses about the distensibility of the airways tree and the structure-function relationship of the lung. This may be a valuable tool for investigators aiming to quantify the influence of airways mechanics *versus* lung compliance and elastance on pulmonary function in healthy and diseased populations[9-11]. Furthermore, accounting for the absolute lung volume at which the volumetric capnographic measurements are being performed allows investigators to characterize the effects of conditions that can alter the inflation state of the lung, such as obesity, lung transplant, or interventions like chest wall strapping. Volumetric capnography may ultimately have clinical utility in the intensive care setting[12,13].

PROTOCOL

This protocol has been previously approved by and follows the guidelines set by the University of Iowa Institutional Review Board. Data shown were collected as part of a project approved by the Institutional Review Board at the University of Iowa. Participants gave informed consent and the studies were performed in accordance with the Declaration of Helsinki.

1. Equipment

1.1. Check the equipment table to verify that all required equipment is available. Double check the configuration using the graphic depiction of the equipment in **Figure 2**.

2. Plethysmography

Note: Barometric plethysmography is a well-described clinical tool and is performed using commercial equipment according to the consensus statements on standardizing lung volume measurements[14,15]. When necessary, lung flows and volumes are compared to predicted values from the NHANES data set and Goldman and Becklake[16] that are included in the plethysmograph software.

2.1. Perform calibration of the plethysmograph daily and prior to any experiments.

2.1.1. Measure the temperature, barometric pressure and relative humidity using a standard barometer prior to the calibration and enter these values into the plethysmograph software as correction factors.

2.1.2. Calibrate the flow sensor using a calibrated 3 L syringe at variable flow rates. Calibrate the box pressure using a precise 50 mL pump. Box pressure transducers should be checked monthly and re-calibrated as needed, per manufacturer's recommendation.

2.2 Immediately prior to the measurement, place the participant in the whole body plethysmograph and close the door. Make measurements after 30-60 s, which allows for thermal equilibration.

2.2.1 Instruct the participant to place their mouth on the mouthpiece, put on nose clips, and place their hands on their cheeks. Preventing "puffing" of the cheeks during the maneuver minimizes changes in volume that result from changing the mouth volume.

2.2.2 Instruct the participant to breathe normally, allowing at least four tidal breaths to be acquired and functional residual capacity (FRC) to be established.

2.2.3. At the end of a normal exhalation (FRC), close the shutter. Coach the participant to pant lightly at 0.5-1 breaths/s for 3-4 s. Evaluate the relationship between the mouth pressure and plethysmograph pressure to ensure that it is a series of overlapping, straight lines without thermal drift.

2.2.4. Open the shutter and allow the participant to take a normal breath. Coach the participant to exhale to residual volume (RV), followed by a maximal inspiratory maneuver to total lung capacity. Repeat at least three times until FRC values that agree within 5% are obtained

3. Volumetric Capnography

Note: Steps 3.1 – 3.4 are performed before the arrival of the research subject.

3.1. Before proceeding, address the variables in **Table 1** and modify if needed. It is important that these variables are adjusted during the study design phase and then held constant for the duration of the study.

3.1.1. Before beginning a new experimental protocol, take care to accurately measure the time delay between the gas analyzer, which measures CO_2 concentration, and the pneumotach, which measures flow. This allows for the CO_2 and flow signals to be aligned.

3.1.2. Measure the time delay experimentally with a stream of 5% CO_2. Attach the gas line to a stopcock, followed by the mouthpiece.

3.1.3 Open the stopcock, introducing the gas at a rate of 10 L/min. Determine the mean time delay between the response of the pneumotach and gas analyzer over 10 trials and enter into the macro.

3.1.4. Maintain the time delay constant by maintaining the analyzer sampling rate. The time delay is highly dependent on the sampling rate of the gas analyzer and it is critical that this remain constant through the experiment and between participants.

3.2 Define three "channels" for the collection of flow, exhaled CO_2 (%), and volume. Flow and exhaled CO_2 (%) are analog inputs and volume is the integral of flow.

3.2.1. Confirm that flow and CO_2 (%) are measured directly from the pneumotach and gas analyzer and that volume is calculated as the integral of flow. **Figure 3** shows that these are being collected in channels 1,2, and 6.

3.3. Calibrate the gas analyzer prior to each use. Include the O_2 sensor if this is to be measured.

3.3.1 Zero the analyzer with an inert gas. 100% calibration grade (<0.01% contaminant) N_2 or He may be used, although helium is preferred because nitrogen may be contaminated with trace amounts of oxygen. Place the drying tube in a bag or connect to a mixing chamber. Flush the bag or chamber with inert gas at a rate of at least 10 L/min. Care should be taken not to pressurize the system as this can impact the calibration.

3.3.2. Flood the bag or chamber with inert gas to displace O_2 and the CO_2. Once the displayed concentrations of CO_2 and O_2 stabilize, adjust the zero knobs until they both read zero.

3.3.3. Repeat with 6% CO_2 and room air (20.93% O_2) as calibration gases. When the concentration of the desired gas stabilizes, adjust the span knob to match the concentration of the calibration gas.

3.3.4. Recheck the inert gas and calibration gases and adjust the zero and span until both are accurate ±0.1%.

3.4. Calibrate the heated pneumotach according to the manufacturer's instructions.

3.4.1 Briefly, allow the pneumotach to warm to 37 °C for at least 20 min prior to the study.

3.4.2 Select the drop-down menu of the flow channel (Channel 1), select the **Spirometer** menu option, and click **Zero** to zero the pneumotach. Finish by selecting **Okay**.

3.4.3. Directly connect a 3L syringe to the pneumotach using a flow head adapter. Highlight the calibration breath. Again, select the drop-down menu of the flow channel. Select **Spirometer flow** | **Calibrate**, type in 3L, and select **Okay**"\.

3.4.4. Check the calibration by injecting 3L into the pneumotach at varying flow rates (0-4 L/s, 4-8 L/s, and 8-12 L/s). The difference from 3 L should be less than 5%.

3.5 Collect the maneuver, ensuring that two sequential breaths are collected and that they are made at the same flow rate.

3.5.1. Coach the subject to perform a single maneuver consisting of two pairs of breaths – a coaching breath and a breath for analysis. This is shown graphically in **Figure 1** (bottom).

3.5.2 During the maneuver, coach the participants to follow the flow guide on the computer monitor. The investigator may coach the subject by indicating “inhale now” or “exhale now”.

3.5.3. Perform the maneuver so that there are two pairs of these breaths in a single maneuver. The first exhalation of the maneuver is 3 s and the second is 5 s. Consider adding a resistor in-line with the mouthpiece in order to make exhaled flow easier to control. A resistance with 5 cm H_2O/L/s of resistance is generally well-tolerated.

Note: It is important that if a resistor is used, it is used throughout the study and for every participant because it increases mouth and airway pressure, which can change airway diameter. It is also important that participants not “puff out” their cheeks as this increases the dead space.

3.6 Measurement protocol

3.6.1 Instruct the participant to sit straight with both feet on the floor, put nose clips on their nose and place their mouth on the mouthpiece.

3.6.2. Coach the participant to complete at least one minute of tidal breathing. This is for measures of metabolic function and allows the participant to familiarize themselves with the mouthpiece. After one minute, stop data collection.

3.6.3. Next, coach the participants to vary their tidal volume, taking either normal, smaller- or larger than normal tidal breaths. This ensures that the capnograms are obtained at different lung volumes

3.6.4 Coach the participant that they should transition to performing a capnogram maneuver as soon as they see the flow tracing appear on their screen.

3.6.5. Resume data collection at a random point in the participant’s respiratory cycle. This allows for measurements to be made at different lung volumes.

3.6.6. Finally, coach to perform a sigh at the end of each maneuver, completely relaxing the muscles of respiration. This allows for FRC to be determined.

3.6.7 Stop data collection. Repeat Steps 3.6.3-3.6.5 until at least 6-8 maneuvers (12 -16 pairs of breaths for analysis) are completed.

4. Data Analysis

4.1 Exporting Data. To run through the macro, each pair of breaths must be exported as a single text file that is then imported into the macro. Screen shots of this process are given in Supplemental **Figure** 1.

4.1.1 Highlight each pair of breaths, taking to care to highlight a portion of the exhalation before the maneuver begins.

4.1.2. Under the file menu, select **Export**, and name the subject's maneuver.

4.1.3. Use the drop-down menu under **Save As Type** and save it as a data file. Then select **Save**.

4.1.4 This will prompt an **Export As Text** box to appear. On the right deselect **Block header Columns, Time, Date, Comments, and Event Markers**.

4.1.5 On the left, select **Current Selection** and **Output NaN for Values**. Select **Downsample by** and enter **10** into the box.

4.1.6 Select the **Flow Channel** and the **CO_2 (%) Channel** to be exported and click O**kay**. Consider making duplicates of these exported files as backups before beginning the analysis.

4.2 Perform the macro analysis. The Step-by-step annotated screen shots of for analyzing exported maneuvers with the macro and comparing to lung volume are given in Supplemental **Figure** 2 and may be used as a guide.

4.2.1 Open the macro, go to file, and select O**pen**.

4.2.2 Select the saved data file, saved with the .txt extension.

4.2.3. A **Text Import Wizard** box will appear. In the upper left-hand corner, select **Delimited** and click **Next**. For step 2, select **Tab** under **Delimiters** and click **Next**. For step 3, select **General** under **Column Data Format** and click **Finish**.

4.2.4. To run the macro, select **View, Macro, View Macro, and Run** in succession. Select **Yes** if there is a backup copy of the data.

4.2.5. Allow the macro to run (approximately 90 s) and generate a workbook with four sheets. Of relevance to these measurements, Sheet 2 contains the numeric data and Chart 3 contains a plot of the capnogram.

4.2.6. Return to the data and determine the volume for FRC. This is identified as the volume at the end of the sigh at which flow = 0 L/s.

4.2.7. Determine the volume at which the second exhalation in each pair of breaths was begun. By subtracting this from the FRC volume, the starting volume above or below FRC can be determined for each breath.

REPRESENTATIVE RESULTS

Representative plethysmography results are given in **Figure 4**. This participant required four attempts in order to collect three FRC values with <5% variability from the mean.%Ref reflects the percent of the predicted value for each variable based on population regression equations that take into account sex, age, race, height and weight

Figure 1 (top) shows a representative single capnogram used in analysis and **Figure 1** (bottom) shows the raw data of the entire sequence of the maneuver. In **Figure 1** (bottom), the capnogram and flow tracing are not aligned to account for the time delay. Data generated from running a sequence of breaths through the macro are shown at the end of **Supplemental Figure 2**. This individual had a dead space of 0.266 L, a slope of 0.523% CO_2/L and a normalized slope of 0.0826 L^{-1}. Quality information about the maneuver are also given in columns F, G, I, J, and K. Column F gives the average exhaled flow rate, with the standard deviation in column G. The exhaled tidal volume is given in column J and the R-squared value for the slope is in column K.

Dead space and slope plotted as a function of lung volume are given in **Figure 5**. In the left panels, dead space and slope are plotted *versus* lung volume relative to FRC, where FRC=0 L. In the right panels, lung volume and slope are plotted *versus* absolute lung volume. In both cases, dead space and slope are significantly correlated to lung volume ($p<0.05$ for all four regression analyses). This suggests that dead space and airways homogeneity increase as lung volume increases, although little is known about this relationship in populations with lung disease or with bronchodilator therapy. The investigator may also choose to use these data to describe the numerical value of dead space and slope at specific lung volumes (FRC, residual volume, 50% of total lung capacity, *etc.*)[3].

FIGURE LEGENDS

Figure 1. Sample capnogram (top), with exhaled CO_2 (%) plotted as a function of the exhaled volume. I, II, and III indicate the three phases of the capnogram. The dotted line indicates the volume of the dead space and the solid line represents the slope of the alveolar plateau (Phase III). The slope can be divided by the area under the capnogram (shaded grey, labeled A) to yield the normalized slope. The four breath sequence is shown in the bottom panel, followed by a sigh breath to determine functional residual capacity. Each pair of breaths is analyzed as a single maneuver.

Figure 2. Equipment setup for capnographic measurements. Shown in this figure are the pneumotach and gas analyzer required for capnographic measurements. The left monitor and

tracing are used by the participant as a guide in generating the flow pattern while data are observed on the right monitor by the investigator.

Figure 3. Channel settings for the acquisition of the volumetric capnogram. Flow is collected in Channel 1, CO_2 concentration (%) is collected in Channel 2, and the tidal volume is calculated in Channel 3.

Figure 4. Representative plethysmograph data from a healthy, male subject. Particularly relevant to the protocol reported here are the total lung capacity (TLC), residual volume (RV) and functional residual capacity (FRC).

Figure 5. Dead space and alveolar slope plotted as a function of absolute lung volume (right panels) and as the volume relative to the functional residual capacity (volume-FRC, left). Note the dependence of the airways volume and lung heterogeneity on lung volume. Lung volume may be expressed as a function of FRC or absolute volume, depending on the experimental design.

Figure 6. Factors impacting data accuracy. Data are given as the mean ± 95% confidence interval. Relationship between the CO_2 sampling rate and the time delay between the gas analyzer and pneumotach (top). The time delay should accurately be determined before beginning the experiment. Measuring eight total maneuvers allows for the measurement of the dead space at a single lung volume with <5% variability (bottom).

DISCUSSION

Here, a protocol for the measurement of V_D and airways homogeneity (slope) is provided. These measurements can be made at FRC, or as a function of lung volume. Measuring FRC before the start of the experiment and after a perturbation allows V_D and slope to be plotted as a function of lung volume and may provide useful information about the structure-function relationship of the lung that is not obtained from capnography at FRC alone.

Airways volume and high-resolution structure can be obtained from computed tomographic imaging[17,18], but this requires exposure to radiation and expertise in image processing. With volumetric capnography, repeated measures can be made without increasing risk to the participant. It also does not require expensive equipment or advanced data processing capabilities. Volumetric capnography is an ideal method for experiments with multiple time points and multiple lung volumes and in-patient populations whose radiation exposure should be minimized.

With regard to the barometric plethysmography, care should be taken to perform the measurement according to consensus statements. When it is important to compare participant values to predicted population values, weight should be measured with a scale and height should be verified with a stadiometer. As noted in the protocol, the most critical component to measure before beginning volumetric capnography is the time delay between the pneumotach and the gas analyzer. The time delay is highly dependent on the analyzer sampling rate (**Figure**

5, top) and small changes in the sampling rate can have large influences on measured values. The analyzer flow rate should be checked at the beginning and throughout the experiment. Calibration of the analyzer and pneumotach are also critical and care should be taken to ensure their accuracy before beginning an experiment.

We have also determined the accuracy of the measurement at a single lung volume in 3 participants. **Figure 5** (bottom) demonstrates that it is necessary to complete four maneuvers (8 total breaths) at a single lung volume to measure dead space so that the variation is <5%. Investigators should take care to make a sufficient number of measurements when having data at a particular lung volume is important. In a subset of 36 maneuvers analyzed in duplicate by two investigators, intra-investigator analysis variability was less than 0.5%.

These methods also require a technician or investigator that is skilled in coaching the participant to make the ventilatory maneuvers. A limitation in pulmonary function studies can be the participant's ability to perform the maneuver. However, participants that are able to perform clinical pulmonary function are typically able to perform the capnographic maneuvers. If the study is designed such that capnography follows plethysmography and spirometry, participants that are unable to perform a coached spirometric or plethysmographic maneuver can be excluded. In 60 previous studies, one participant who performed clinical spirometry was excluded because they could not follow the capnographic breathing pattern. There are currently no consensus guidelines defining acceptable capnographic measurement criteria. However, intersubject variability is 8±1% of the target flow rate in our 10 most recent participants. Intrasubject (between maneuver) variability is 4±2%.

Issues relating to data accuracy and reproducibility are the result of errors in the time delay or the analyzer and pneumotach calibration. Before each experiment, take care to calibrate the analyzer with a set of known gases and generate a multi-point standard curve to confirm the analyzer's accuracy.

Beyond the scope of the information provided here, the macro contains two additional calculations that may be of interest. When the maneuvers are made at FRC, the FRC column provides an estimate of FRC based on the Farmery method[19]. Calculation of the peripheral bronchial cross sectional area is based on the method described by Scherer, *et al.*[20]. Finally, if desired, the end tidal CO_2 and average expired CO_2 concentration can be used to calculate the physiological dead space for comparison to the anatomic dead space[21,22].

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DISCLOSURES

The authors have nothing to disclose.

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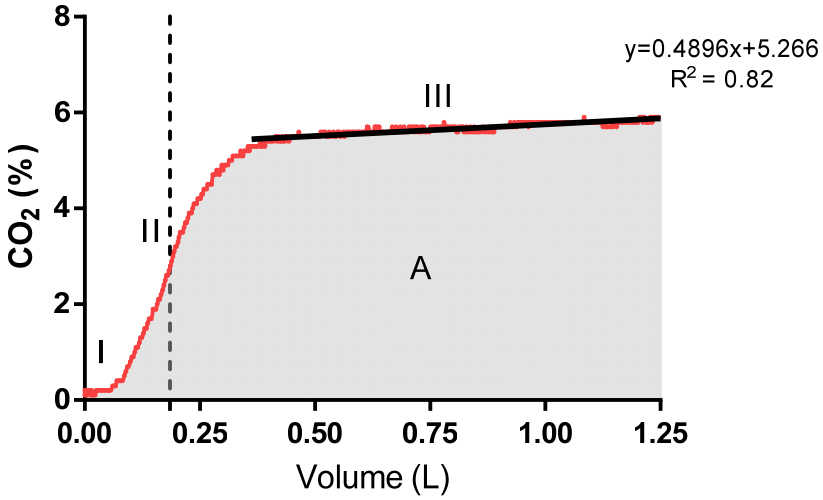

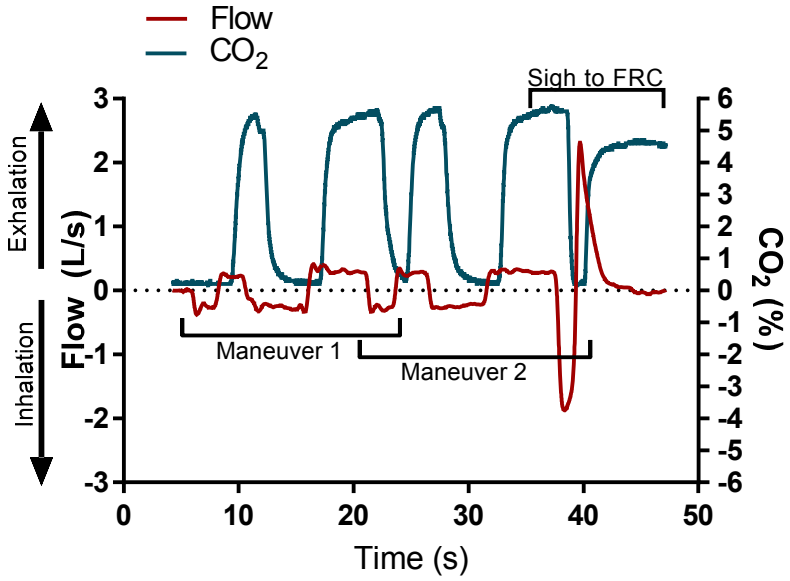

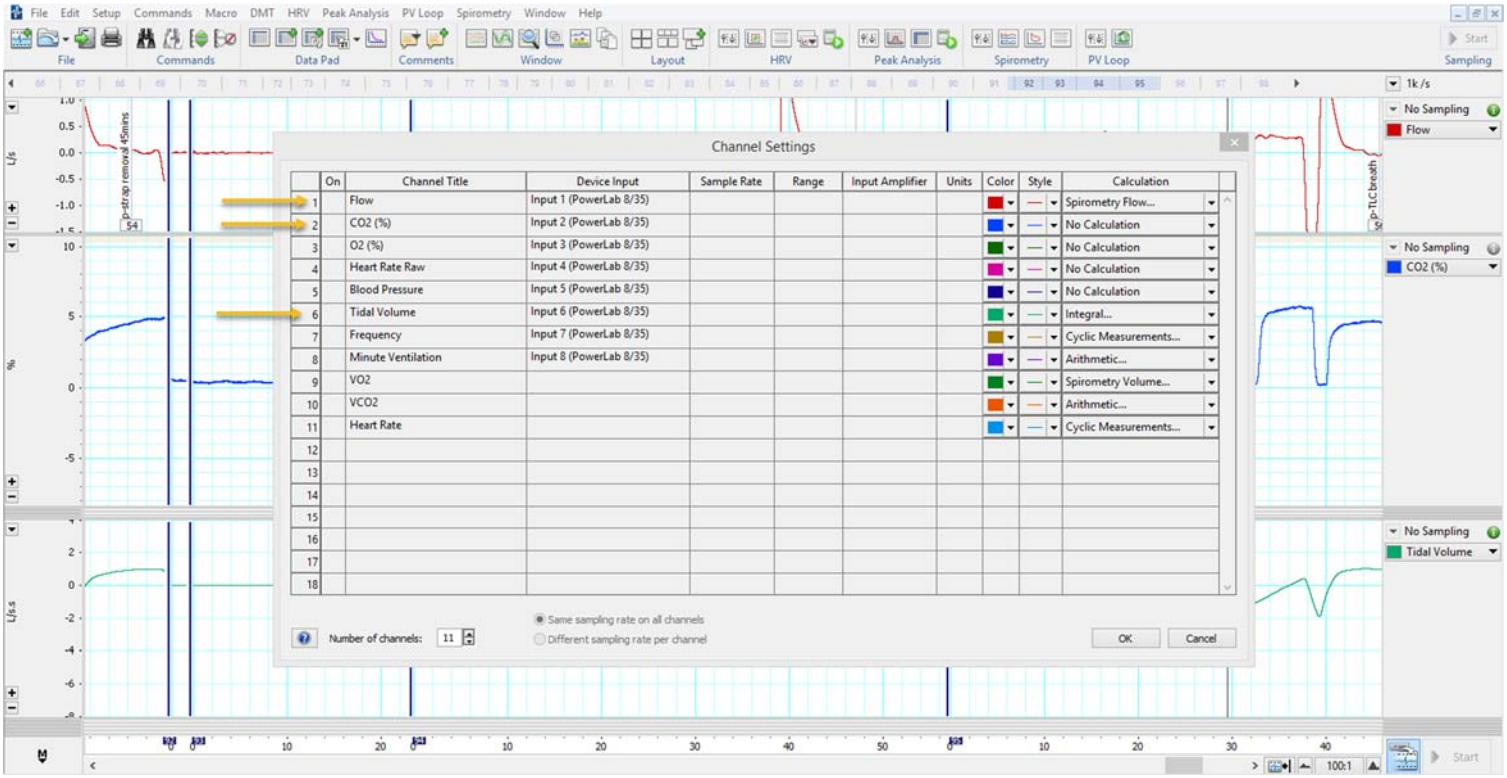

Pulmonary and Exercise Physiology Lab
Clinical Research Unit, Boyd Tower 2110
University of Iowa
Iowa City, IA

Date: 02/01/18

Plethysmography ---

	Ref	Best	% Ref	1	2	3
TLC	8.05	7.61	95	7.39	7.44	7.64
Vtg		3.26		3.17	3.37	3.25
RV/TLC	27	18		17	17	18
RV	2.15	1.36	63	1.29	1.27	1.39
ERV		1.89		2.02	1.77	1.89
FRC PL	4.28	3.21	75	3.31	3.04	3.28
VC	6.16	6.25	102	6.10	6.17	6.25
IC		4.40		4.08	4.40	4.36
Vtg f		55		78	38	48
sGaw	0.232					
Raw	1.01					
sRaw						
Vt		2.04		3.74	1.09	1.27
LVol ECode		000000		00	00	00
LVol Time		12:45		12:45	12:46	12:48
f		16			17	15
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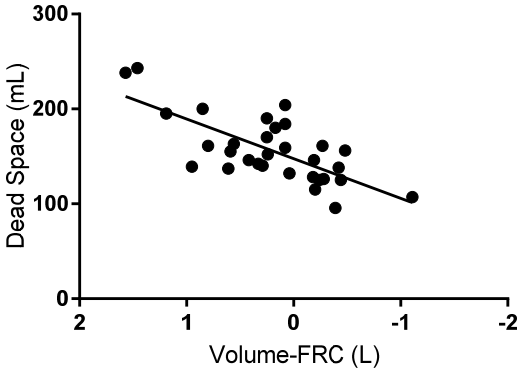

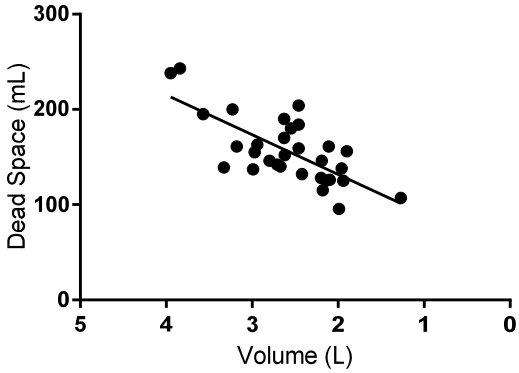

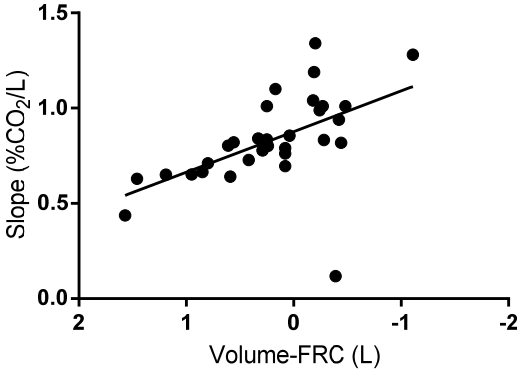

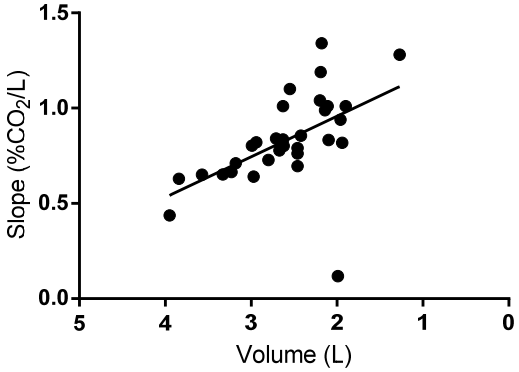

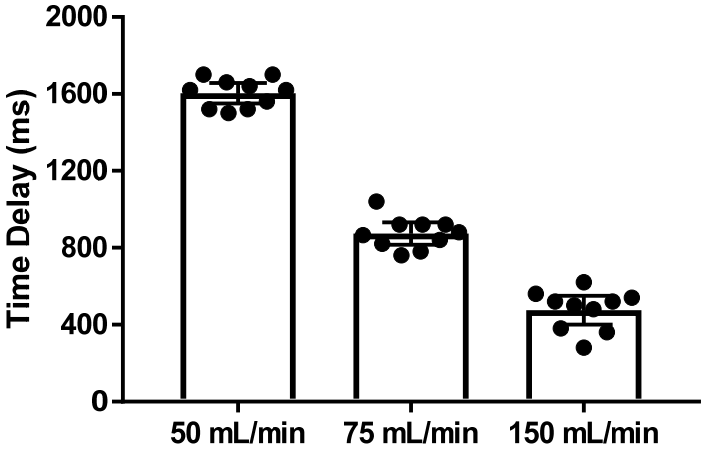

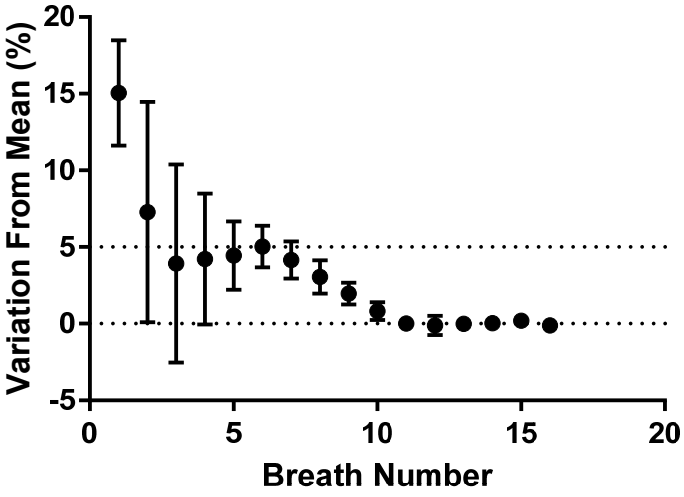

Table 1. Macro variables that may be changed prior to data analysis

Const targetFlow	This is the target flow rate at which the participant is coached to exhale in mL/s. In previous studies, we have coached participants to breath at 400 mL/s. In more recent studies of patients with expiratory flow limitation, we have found it necessary to reduce this to 250 mL/s.
Const Delay	This is the time delay between the gas analyzer and the pneumotach. Measuring this is critically important as this value will depend on the gas sampling rate and length of the sampling tube.
Const Calconst	This parameter provides a correction for pneumotach calibration. We previously collected data from the pneumotach in volts and used a calibration factor to convert to L/min. We now calibrate our pneumotach in L/min in Powerlab, so the calibration constant is set to 1.
Const samprate	The default value of this is 100 Hz. Data is collected in our lab at 1000 Hz, but down sampled by a factor of 10 during data export in order to minimize file size and processing time.
Const volumeCap	This is the volume at which the second breath is truncated for analysis. We set the default value to 1250 mL to ensure that we are sampling alveolar gas and collect at least two times the dead space, but do not reach closing volume. This may need to be adjusted for patients with smaller lung or closing volumes.

Note: These terms are also highlighted in the macro, provided in the supplemental materials

Materials Table

Click here to access/download;Table of Materials;JoVE_Materials Seymour.xlsx

Name of Material/ Equipment	Company	Catalog Number
Computer with dual monitor	Dell Instruments	
PowerLab 8/35*	AD Instruments	PL3508
LabChart Data Acquisition Software*	AD Instruments	Version 8
Gemini Respiratory Gas Analyzer* (upgraded option)	CWE, Inc	GEMINI 14-10000
Heated Pneumotach with Heater Controller* (upgraded option)	Hans Rudolph, Inc	MLT3813H-V
3L Calibration Syringe	Vitalograph	36020
Nose Clip*	VacuMed	Snuffer 1008
Pulse Transducer*	AD Instruments	TN1012/ST
Barometer	Fischer Scientific	15-078-198
Flanged Mouthpiece*	AD Instruments	MLA1026
Nafion drying tube with three-way stopcock*	AD Instruments	MLA0343
Desiccant cartridge (optional for humid environments)*	AD Instruments	MLA6024
Resistor	Hans Rudolph, Inc	7100 R5
Flow head adapters*	AD Instruments	MLA1081
Modified Tubing Adapter (optional)	AD Instruments	SP0145
Two way non-rebreather valve (optional)*	AD Instruments	SP0146
Plethysmograph	Vyaire	V62J
High Purity Helium Gas	Praxair	He 4.8
6% CO_2 and 16% O_2 Calibration Gas	Praxair	Custom

Microsoft Excel Microsoft Office 365

Comments/Description

*indicates that part is available in the Exercise Physiology package from AD Instruments

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Title of Article:	Combining volumetric capnography and barometric plethysmography to measure the lung structure-function relationship
Author(s):	Seymour, Pritchard, Sajjad, Tomasson, Blodgett, Winnike, Paun, Eberlein and Bates

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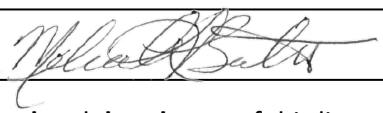
Signature:	
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10. Figure 6: Please define the error bar
11. Please remove all headers in Discussion.
12. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). Any text that cannot be written in the imperative tense may be added as a "Note."
13. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
14. Please add a one-line space between each of your protocol steps.
15. Please do no highlight notes for filming.
16. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.

We believe that these have been addressed.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
In this manuscript, the authors describe a robust method for measuring anatomic dead space and for representing this important anatomic/physiologic parameter more truly as a variable dependent upon lung volume. The method described is detailed, robust, and can be performed with readily available equipment such that it could be employed in many existing pulmonary function labs.

We appreciate the reviewer's encouragement and have tried to address their comments to the best of our ability.

Major Concerns:
For this reviewer, two issues could be addressed that might significantly improve the reader/end-user experience
1. A more detailed discussion of some of the common pitfalls, particularly with regard to subject understanding and performance, and how these issues are handled by authors would be very helpful. In this reviewer's experience, at least a significant minority of people will have some difficulty understanding and following instructions in routine pulmonary function testing performed for clinical purposes. The protocol described sounds like it might be a step more complex than that. How often do subjects have trouble with the maneuvers? Do the authors have a standard "script" or coaching approach

We thank this reviewer for encouraging us to expand this section. We agree that coaching individuals to perform pulmonary function maneuvers can be quite challenging and have added some detail about our study workflow and why we might exclude a participant. There are no current guidelines for capnography (as there are for pulmonary function), so we retrospectively quantified precision in our past 10 subjects in order to give the reader an idea about the degree of intrasubject and intersubject variability they can expect. We do coach participants when to breathe in and out and have added this information, but having the screen template to follow is very helpful and the vast majority of individuals can follow the breathing pattern. In our experience, performing plethysmography is more challenging.

2. If the authors have any data showing how their measurements/methods perform in the face of a perturbation to the system that might affect airways volume - e.g., pre and post bronchodilator administration, or in the context of a methacholine challenge, or something like that - it would be of real utility to the reader who is trying to decide whether to utilize these methods and whether there would be sufficient sensitivity to test the potential user's hypotheses.

We appreciate this comment and determining the sensitivity of this measurement is a major subject of our current investigation. We hope to complete and publish our results later this year. For the sake of transparency, we have added this.

Minor Concerns:
1. How does the method perform in settings of perturbed CO2 handling (e.g., COPD with CO2 retention, obesity hypoventilation syndrome, etc.)?

This is also not well-known. We have not made these measurements in patients that are hypoxemic and or retaining CO2, but this is an important area of investigation.

2. The authors state that either nitrogen or helium can be used for inert gas calibration. Are they equivalent? This reviewer asks only because the boiling points of nitrogen and oxygen are such that nitrogen can be contaminated by a small amount of oxygen, whereas the noble gases like helium or argon typically do not have this issue.

We agree and have added that calibration gas must be used where the contamination is verified as <0.01%. We use He and it is the preferred gas, although we know that some of our collaborators find obtaining He to be challenging and include N2 as an alternative. Given that the sensitivity of our analyzer is 0.1%, this is reasonable.

3. A quick read-through for grammatical hiccups would be great. There are a few places where there are phrasings like "A steeper slope of Phase III suggests a less uniform the airway tree proximal to the terminal bronchioles, or convection-dependent inhomogeneity (20)." (lines 95 and 96). Very minor things, but may distract the distractible reader.

Thank you for the careful read and the helpful comments

Reviewer #2:
Manuscript Summary:

The authors propose here their rationale and a well-constructed protocol to combine volumetric capnography and barometric plethysmography in order to assess both the anatomic deadspace and lung volume and compliance. The authors also provide a macro to analyze capnographic data.
Although these two methods have previously been described and used for along time, the authors submit here a clear description of a smart combination of both, including how to assemble it, use it and analyze it in patients. This technique will undoubtedly be of important interest to researchers from the respiratory field.

We also appreciate this reviewer's encouraging comments and hope that our colleagues find this methods paper useful.

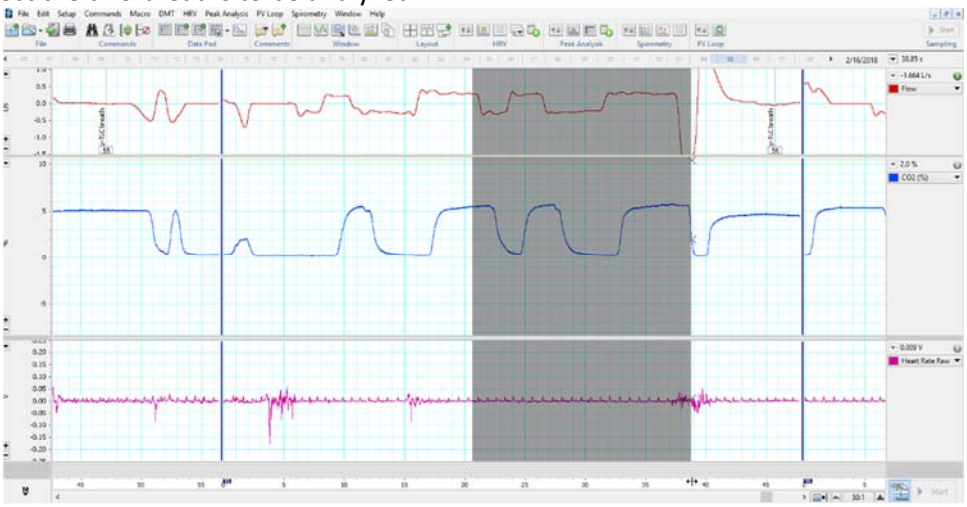
Major Concerns:
I have no major concern. The manuscript and the method are very clear, and I think that the intro and discussion fit very well into the manuscript.

Thank you.

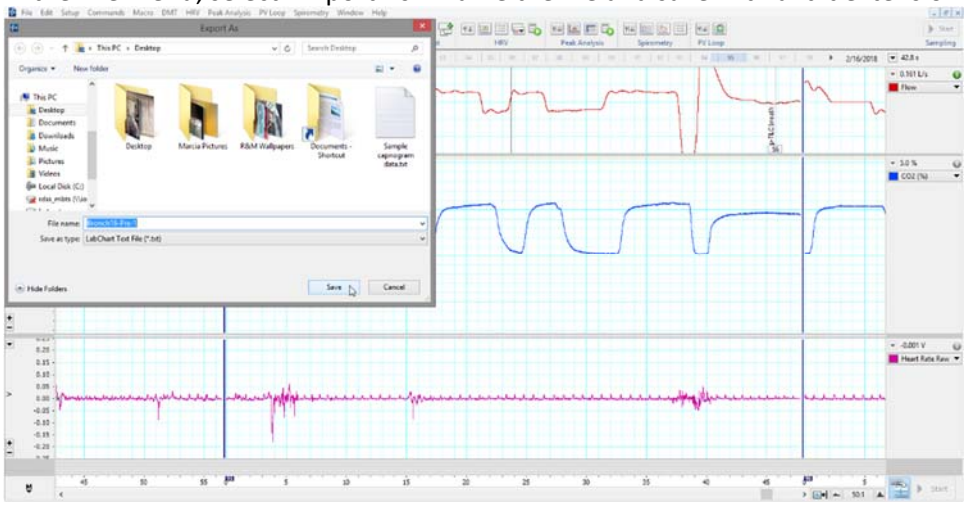
Minor Concerns:
Page 4, line 114: replace "capnorgraphy" with "capnography"

This is completed.

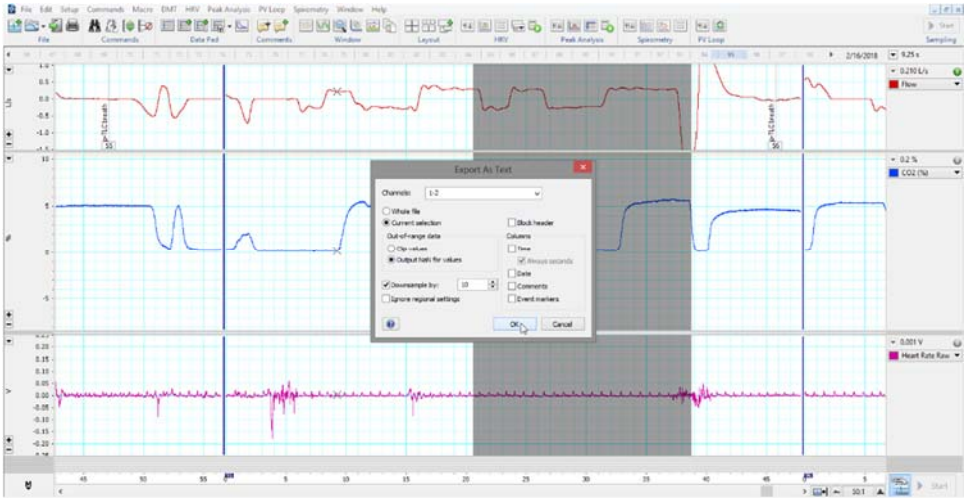
Step 1: Select the two breaths to be analyzed.



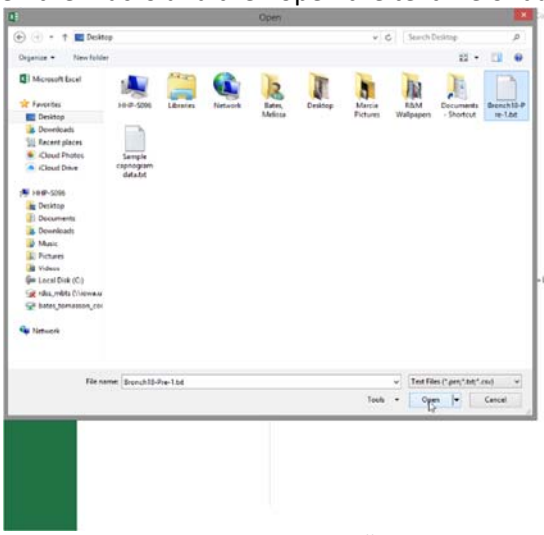
Step 2: From the File menu, select “Export As.” Name the file and save with a .txt extension.



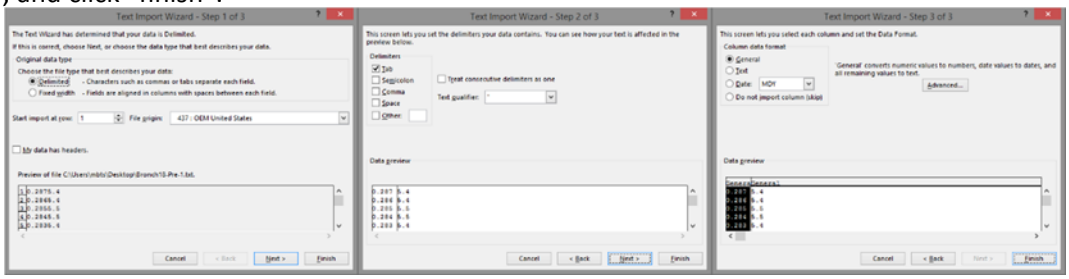
Step 3: Select the flow and CO_2 channels to export, check “current selection” and downsample by a factor of 10



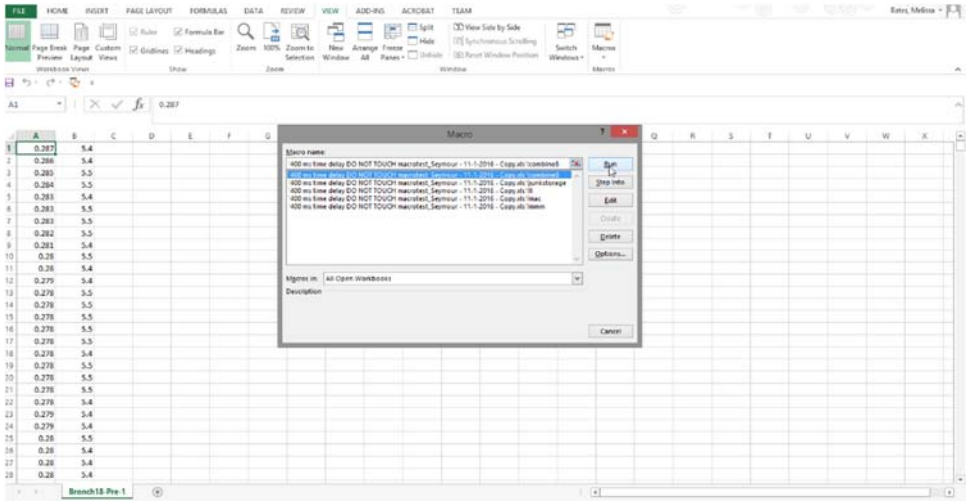
Step 1: Open the macro and then open the text file of data



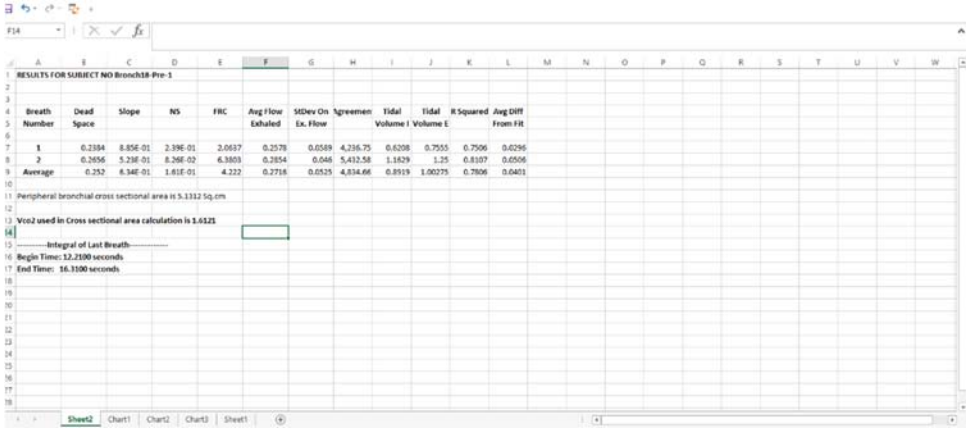
Step 2: In the Text Import Wizard, select “delimited data” and click “next”. Select “tab” and click “next”, and click “finish”.



Step 3: Select the first macro file and click “Run”



Step 4: Summarized data will be populated to Sheet 2. Data for breath #1 should be discarded.



RESULTS FOR SUBJECT NO Bronch18-Pre-1

Breath Number	Dead Space	Slope	NS	FRC	Avg Flow Exhaled	StDev On Ex. Flow	%reemen	Tidal Volume I	Tidal Volume E	R Squared	Avg Diff From Fit
1	0.2384	8.85E-01	2.39E-01	2.0637	0.2578	0.0589	4,236.75	0.6208	0.7555	0.7506	0.0296
2	0.2656	5.23E-01	8.26E-02	6.3803	0.2854	0.046	5,432.58	1.1629	1.25	0.8107	0.0506
Average	0.252	6.34E-01	1.61E-01	4.222	0.2716	0.0525	4,834.66	0.8919	1.00275	0.7806	0.0401

Peripheral bronchial cross sectional area is 5.1312 Sq.cm

Vco2 used in Cross sectional area calculation is 1.6121

----------Integral of Last Breath----------

Begin Time: 12.2100 seconds

End Time: 16.3100 seconds

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Supplemental Coding Files

Macro code - Supplemental Material.docx