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Measuring Breathing Patterns in Mice Using Whole-body Plethysmography

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[Abstract] Respiratory dysfunction is among the main cause of severe and fatal pathologies worldwide. The use of effective experimental models and methodologies for the study of the pulmonary pathophysiology is necessary to prevent, control and cure these diseases. Plethysmography, a technique for the assessment of lung function, has been widely applied in mice for the characterization of respiratory physiology. However, classical plethysmography methods present technical limitations such as the use of anesthesia and animal immobilization. Whole-body plethysmography (WBP) avoids these issues providing a non-invasive approach for the assessment of the respiratory function in conscious animals. WBP relies on the recording of pressure changes that are produced by the spontaneous breathing activity of an animal placed inside an airtight chamber. During normal respiration, pressure variation is directly proportional to the respiratory pattern of the animal allowing the measurement of the respiratory rate and tidal volume. These parameters are commonly used to evaluate pulmonary function in different physiological and disease models. In contrast to classical plethysmography methods, WBP technique allows reproducible serial measurements as it avoids animal restraint or the use of anesthesia. These key features rend WBP a suitable approach for longitudinal studies allowing the assessment of progressive respiratory alterations in physiological and pathological conditions. This protocol describes the procedures for the measurement of the breathing patterns in mice using the WBP method, the data analysis and results interpretation.

Keywords: Whole-body Plethysmography, Tidal Volume, Breathing, Respiration, Mouse, Non-invasive, Conscious, Unrestrained

[Background] Respiratory diseases are leading causes of disability and the third cause of death worldwide. To address the prevention, control and cure of these diseases basic research in the field is needed (Forum of International Respiratory Societies, 2017). Animal models, such as murine models, have been very useful in research due to a well-characterized genome, an improvement in genetically engineered animals, short breeding cycle, cost-efficiency and a well-known immunologic system (Gelfand, 2002; Persson, 2002; Irvin and Bates, 2003). Despite their small size, mice have contributed to the understanding of lung mechanics and functioning in normal conditions as well as in disease (Bates, 2017).

Whole-body plethysmography (WBP) is a technique that allows the analysis of the respiratory function.

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It is a non-invasive, precise approach that does not required anesthesia, as the mouse is unrestrained and conscious. Therefore, the respiratory parameters obtained with WBP in mice reflect basal physiological values since instrumental restraints and/or anesthesia are not applied (Lim *et al.*, 2014; Quindry *et al*., 2016). Notably, these experimental conditions are key for the use of WBP in longitudinal studies (Cramer *et al.*, 2015; Flanagan *et al*., 2019). Moreover, WBP in mice is a well-stablished technique that has been applied for the study of a wide range of biological aspects of the respiratory function providing new insights into neuronal network controlling respiratory rhythm (Crone *et al*., 2012), sleep-related breathing disorders (Bastianini *et al.*, 2017) or the role of inflammation in the control of breathing (Giannakopoulou *et al*., 2019).

During WBP, the mouse is placed in an airtight chamber where it can move freely and breathe spontaneously. Inspiration and expiration cycles modify the chamber pressure due to gas compression and expansion within the lungs as well as to changes in temperature and humidity of the air as it enters into the respiratory tract (Lundblad *et al.*, 2002; Lim *et al.*, 2014; Bates, 2017). Breathing frequency and tidal volume can be measured based on the variations in the chamber pressure parameters (period and magnitude) (Lundblad *et al.*, 2002; Adler *et al.*, 2004; Bates, 2017).

Other conventional plethysmography methods require the animal to be immobilized (Double-chamber plethysmography, DCP), or need invasive surgery in an unconscious mouse (Forced oscillation technique, FOT), which does not allow animal recovery. DCP consists of two chambers that separate animal head and thorax in two chambers (Mailhot-Larouche *et al.*, 2018). Habituation is needed prior to acquisition data due to restraint-induced stress. Restraint is well known as a major stressor and it alters respiration parameters (Buynitsky and Mostofsky, 2009), which may confound result interpretation. Another broadly used approach in lung mechanics is FOT, which requires that the subject's respiratory system remains passive during the mechanical ventilation (McGovern *et al.*, 2013). This is achieved by the administration of anesthetics and tracheotomy. Forced oscillation technique provides reproducible and detailed data due to controlled experimental conditions although these are far from the physiological state (Mailhot-Larouche *et al.*, 2018).

Compared to conventional plethysmography techniques, WBP offers a more versatile and reproducible tool for the assessment of the respiratory function. In addition, body plethysmography is a well-established technique for lung function determination in humans (Criée *et al.*, 2011). Therefore, respiratory diseases in humans, such as asthma and pulmonary fibrosis, can be modeled by whole-body plethysmography in mice (Finkelman, 2008; Vaickus *et al*., 2010; Milton *et al.*, 2012). WBP also enables lung mechanics studies with hypoxic and hyperoxic models, measurement of infectious agents, allergen sensitization studies and anesthetics function (Detweiler *et al.*, 2018; Hill *et al.*, 2018; Ortega-Sáenz *et al.*, 2018; Receno *et al*., 2019), which can be progressively monitored. Moreover, WBP can be applied to study alterations in respiratory function that can be a key clinical sign in diseases with secondary respiratory impairment, such as Leigh Syndrome (Quintana *et al.*, 2012; Bolea *et al.*, 2019).

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Materials and Reagents

1. SilicaGel Orange Indicator 2-4 mm (Bolaseca, catalog number: 8.1.002)
2. Laboratory tissue paper, *e.g.*, Wiping Paper Plus (Tork, catalog number: 130050)
3. C57BL/6J background mice (The Jackson Laboratory, catalog number: 000664)
   Note: Other laboratory mice strains can be used. Please, see Procedure section.
4. Ethanol 70%, *e.g.*, Ethanol absolute for analysis (PanReac AppliChem, catalog number: A1613)

Equipment

1. Plethysmograph, Unrestrained Whole Body (90 mm diameter), for Mouse (EMMS, catalog number: PLY310)
2. Pulmonary Flow Transducer (EMMS, catalog number: TPF100)
3. Adaptive Amplifier, strain gauge type; single channel (EMMS, catalog number: AMP110)
4. Large capacity drying column (0.5 kg indicating Silica desiccant, suitable for regeneration) complete with tubing and fitting, including 1ml Lock syringe connect to a plastic tube (EMMS, catalog number: ADR101)
5. 2-channel Bias Flow Generator (EMMS, catalog number: AIR140)
6. Acquisition Unit (laptop)

Software

1. USB Data Acquisition (eDacq) Single Subject Version 1.9.0 (Site) only version with Flow Derived Parameters Analyser (EMMS, catalog number: ESS101A)
   Note: EMMS company (Electro-Medical Measurement Systems), Bordon, UK, www.electromedsys.com (equipment and eDacq software).
2. Microsoft Office Excel 2016 or compatible
3. Statistical software (*e.g.*, GraphPad Prism 6.0)

Procedure

Several parameters may critically influence WBP outcomes and reproducibility. Therefore, they might be considered in the experimental design. These include time of the day (considering mouse circadian cycle), mouse behavior during recording, and standardization of the habituation period (Quindry *et al.*, 2016; Receno *et al.*, 2019). Depending on the specific research question, strain-specific differences in pulmonary physiology might be evaluated (Cramer *et al.*, 2015; Bates, 2017). Importantly, the parameters described in the following section may be adapted and validated considering the basal/control breathing activity of each mouse strain.

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Furthermore, environmental conditions must be kept constant during the entire procedure. Here, we maintained a constant temperature of 22 °C and a relative humidity of 55% (as recommended in the Guide for the Care and Use of Animals, National Research Council, 2011).

A. Setting up the equipment

1. Install USB Data Acquisition (eDacq) to the computer.
2. Connect through the USB connector the Adaptive Amplifier to the acquisition unit.
3. Attach Pulmonary Flow Transducer into the transducer entrance from Adaptive Amplifier (Figure 1D).
4. Join the Pulmonary Flow Transducer to the Plethysmograph apparatus (Figure 1A).
5. Set up Flow 1 and Flow 2 levels at 1cc/min from the Bias Flow Generator (Figure 1C).
6. Join Port 1 from Bias Flow Generator with Entrance 1 of Large capacity drying column and Port 2 to the top input from Plethysmograph chamber. Then, connect Entrance 2 of drying column to the lower input from Plethysmograph device (Figures 1A and 1C).

Note: Silica beads must be maintained dehydrated, when hydrated (color indicator shifts from orange to green, Figure 1C) it is necessary to replace the beads or reactivate them by drying them at 150 °C for 24 h.

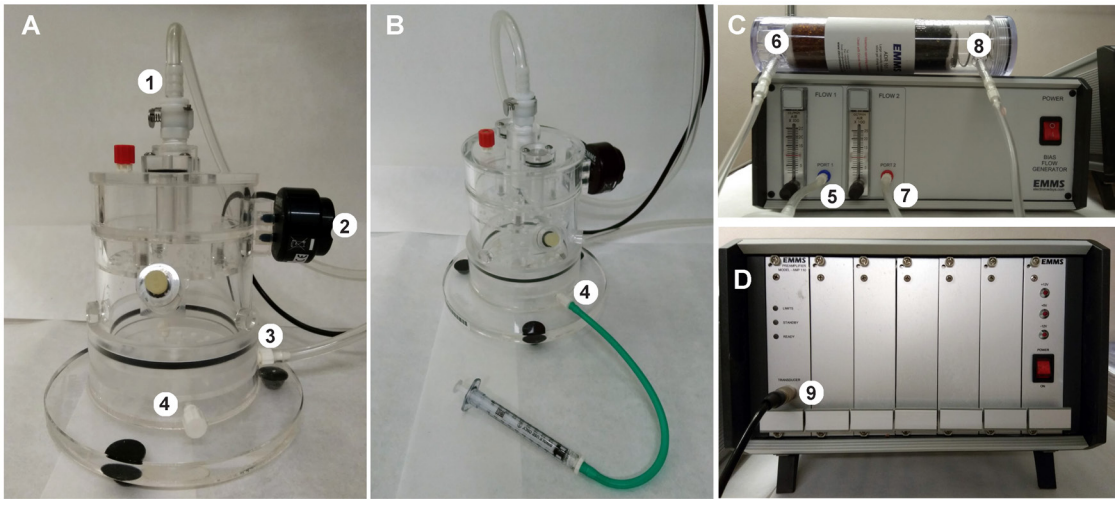

Figure 1. **Plethysmograph equipment.** A. Plethysmograph chamber. 1: Top input (incoming airflow), 2: Pulmonary Flow Transducer, 3: Lower input (air exit), 4: Alternative lower input). B. Plethysmograph apparatus attached to the 1 ml Lock syringe by the alternative lower input (4). C. Bias Flow generator and drying column containing silica beads. 5: Port 1 (flow1), 6: Entrance 1, 7: Port 2 (flow2, incoming air), 8: Entrance 2 (air extraction). D. Adaptive amplifier connected to the chamber through transducer entrance (9). Fresh air is continuously administrated into the chamber trough the Port2 and released from the chamber trough the lower input (3). Then, the air passes through the drying column and it is extracted from the Bias Flow generator (air dehumidification is required to preserve bias flow generator).

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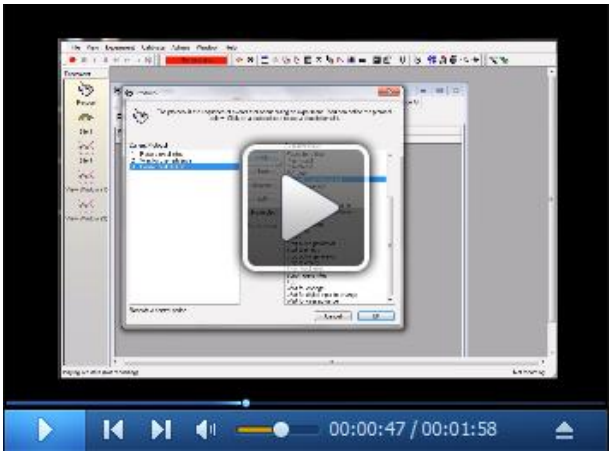
B. Setting up the protocol (see Video 1)

1. Start eDacq program.
2. Select **Protocol** from left panel. A new window will pop up.
3. Click **Site 1** option and then **Edit**.
4. Add and edit the different steps of the desired protocol:
   a. Record on all sites
   b. Wait for user advance
   c. Pause for 00:03:00 (*time to leave the workspace/room*)
   d. Record control period for 00:45:00 (*habituation time*)
   e. Record period for 00:15:00 (*recording time*)
   f. Record off all sites
5. Select Site 1 from left panel. Add the following flow derived parameters (FDP) properties for recording:
   a. On the **General** tab: in the **Input** group select 20 ms of smoothing and in the **Output** group select 5 s of time based.
   b. On the **Breath Analysis** tab: in the **Breath Detection** group introduce 0.5 ml/s for Row Threshold and 20% TV for Start of Breath Extrapolation. In the **Breath Rejection** group, do not select **Enable Breath Rejection**.
   c. On the **Filtering tab**: do not select **Enable-Pass Filter** and **Enable Mains Filter**.
6. Apply all changes.
7. Save configuration.

Note: Experimental parameters (from Steps B4 to B5) may be adjusted depending on the biological question, experimental design, and mouse phenotype. Please check manufacturer's recommendations.



Video 1. Setting up the protocol

C. Chamber calibration (see Video 2)

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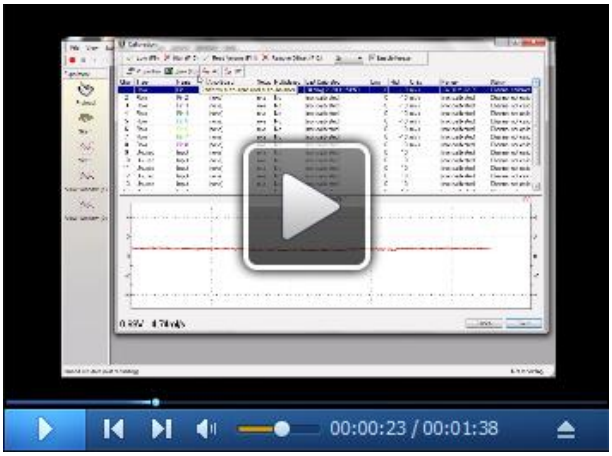
1. Turn on the Bias Flow Generator and the Adaptive Amplifier (a white light will flash on standby) 15 min before starting calibration.
2. Tightly close the plethysmography chamber.
3. Start eDacq program.
4. Introduce assigned password (select ok).
5. Introduce study name (press close).
6. Close the configuration file version window that appears.
7. Accept calibration option (always recommended).
8. Activate **DC mode** for calibration (AC mode is automatically set during recording).
9. Set the high flow value to -10 ml/s.
10. Press **F8** (Zero button) to adjust basal parameters.
11. Press **F9** (Low) to adjust low calibration volume.
12. Join 1ml lock syringe to the alternative lower stopcock input of plethysmography chamber (Figure1 A-B).
13. Gently inject 1 ml air volume into the chamber using the connected syringe and 1.5 s after press **F11** (Record volume) to adjust high calibration volume. Proper calibration range should be between -35 and +35 ml/s (repeat the process otherwise).
14. Remove the syringe and place back the cap.
15. Click **Finish** button and proceed to respiration recording.
16. A white light should flash on ready from the adaptive amplifier machine.

Notes:

a. *Calibration must be performed every time the equipment is switched on. There is no need to calibrate between each animal/trial in the same day. Calibration can also be initiated through the **U** option from toolbar at any time.*

b. *High flow value must be ten times the injected calibration volume (step 13) as a correction factor for FDP analysis.*



Video 2. System calibration

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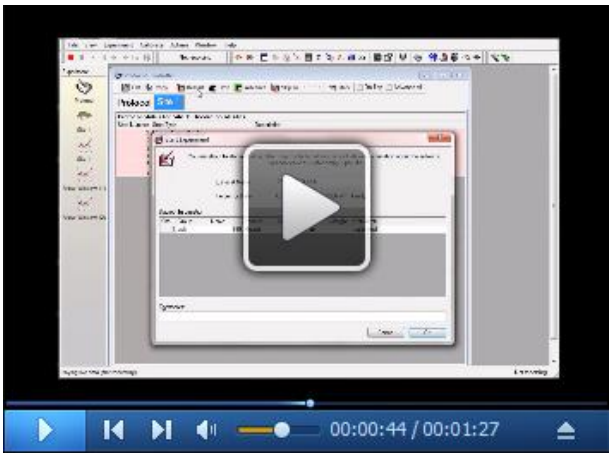
D. Breathing recording (see Video 3)

1. Weigh the mouse right before recording.
2. Open the plethysmography device and place the animal inside.
   Note: It is critical not disturbing the transducer when manipulating the chamber device. Vibrations can affect the calibration. If this occurs recalibrate the system. Avoid installing the equipment in unstable surfaces or close to high-traffic areas.
3. Close the chamber tightly and make sure the animal moves freely.
4. Select **Protocol** option and then **Site 1** choice (the pre-configured protocol will be open).
5. **Restart** the protocol.
6. Introduce mouse identification, weight and any other comments of interest. After assessing this step, recording will start, (green flashing window on screen may appear).
7. **Advance** the protocol to *wait period* (3 min) step. After this period the protocol will automatically proceed to the following steps.
   Note: During this interval we recommend that the user leaves the workspace/room without disturbing the equipment to avoid interferences with the analysis. However, mouse must be monitored by visual resources or from outside the room through the whole session to record motor activity periods as well as any other behaviors.
8. After recording, return the animal to its cage. Clean and wipe the equipment with 70% Ethanol. Recording will be automatically saved in the assigned project folder.
9. Record next animal by restart button (start from Step D1) or proceed to data analysis.
10. Turn off all the equipment.



Video 3. Recording whole-body plethysmography

Data analysis

eDacq software displays the waveforms from the transducers in real-time. Data is finally recorded to a disk file for post experiment replay and analysis. Critically, to avoid background noise during the recording, especially from mouse motor activity, it is crucial to allow sufficient habituation time. Therefore,

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while the breathing patterns of the animal are recorded for 1 h, only data from the final 15 min are analyzed. Importantly, since no breath rejection parameters are applied, it is necessary to ensure data corresponds to resting periods (Figure 2).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	Period	Index	Cur t	Rec t	Rel t	Rel us	s	n	nRej	TV,average (ml)	TV (µL/g)	tI,average (s)	tE,average (s)	PIf,average (ml/s)	PEf,average (ml/s)	f,average (breath/minute)
2																
3	Initial User: Admin (Administrator user) {06A1AB79-EB1E-405D-B994-F5669657381A}															
4	Comments:										Weight mouse (g)					
5	eDacq Version: 1,9,0										13.34					
6	Analyser Version: FDP; v 22															
7	Configuration file used: "C:\Program Files (x86)\EMMS\eDacq\WBP 01 Mouse UAB,ecf"															
8	Configuration file version: 13															
9	Subject name: X															
10	Group: Y	Species: Mouse			Strain: not specified											
11	Sex: M	Weight: 13.34 g			Comments: calibrated											
12	Data Source: Live data				Start time: 28 set 2018 11:43:18											
13	Analyser Settings: General Settings: Analyser version=22; Smoothing=20000us; Enable auto offset=0; Auto offset window=10; Output=Every 25 breaths; Breath Detection: Flow Threshold (ml/s)=0,5;															
14	Start of Breath Extrapolation (%TV)=20; Breath Rejection: Enable Breath Rejection=0; Minimum TV (ml)=0,05; Minimum IT (s)=0,02; Maximum IT (s)=3; Insp/Exp Volume Balance (%)=40;															
15	Minimum Breathing Frequency (bpm)=25; Maximum Breathing Frequency (bpm)=800; Filtering: Enable Filtering=0; Low Frequency (Hz)=0,25; High Frequency (Hz)=5; Enable Mains Filter=0; (Hz)=50;															
16	Audit Trail ID: 249774B1-84B7-4675-A2EF-8D7E2F73E9A8															
17																
18	Data:										=(TV/g)*1000					
19	Control	1790	12:28:12	28/09/2018 12:28	01:49:49	948000	6589	25	0	0.136	10.19	0.05	0.10	4 487	2 138	422.3
20	Control	1791	12:28:16	28/09/2018 12:28	01:49:53	908000	6593	25	0	0.161	12.07	0.05	0.09	4 608	1 964	483.2
21	Control	1792	12:28:19	28/09/2018 12:28	01:49:56	914000	6596	25	0	0.143	10.72	0.05	0.11	4 172	1 623	414.5
22	Control	1793	12:28:22	28/09/2018 12:28	01:50:00	310000	6600	25	0	0.141	10.57	0.05	0.08	4 915	2 203	502.3
23	Period	1794	12:28:26	28/09/2018 12:28	01:50:04	308000	6604	25	0	0.148	**11.09**	0.061	0.11	3 653	1 755	**363.5**
24	Period	1795	12:28:30	28/09/2018 12:28	01:50:08	12000	6608	25	0	0.148	**11.09**	0.049	0.10	4 888	1 870	**427.4**
25	Period	1796	12:28:33	28/09/2018 12:28	01:50:11	12000	6611	25	0	0.141	**10.57**	0.045	0.07	4 752	1 753	**548.3**
26	Period	1797	12:28:37	28/09/2018 12:28	01:50:14	904000	6614	25	0	0.154	**11.54**	0.051	0.08	4 744	1 680	**472.3**
27	Period	1798	12:28:41	28/09/2018 12:28	01:50:19	378000	6619	25	0	0.152	**11.39**	0.058	0.11	4 271	1 691	**354.8**
28	Period	1799	12:28:45	28/09/2018 12:28	01:50:22	594000	6622	25	0	0.154	**11.54**	0.065	0.11	3 830	1 535	**349.4**
29	Period	1800	12:28:48	28/09/2018 12:28	01:50:26	130000	6626	25	0	0.145	**10.87**	0.062	0.11	3 580	1 713	**386.4**
30	Period	1801	12:28:51	28/09/2018 12:28	01:50:29	148000	6629	25	0	0.152	**11.39**	0.056	0.10	4 227	1 695	**394.5**
31	Period	1802	12:28:54	28/09/2018 12:28	01:50:32	58000	6632	25	0	0.128	**9.60**	0.043	0.07	4 434	1 964	**626.3**
32	Period	1803	12:28:57	28/09/2018 12:28	01:50:34	896000	6634	25	0	0.142	**10.64**	0.046	0.07	4 695	2 147	**584.6**
33	Period	1804	12:28:59	28/09/2018 12:28	01:50:37	426000	6637	25	0	0.121	**9.07**	0.041	0.06	4 665	2 101	**683.5**
34	Period	1805	12:29:02	28/09/2018 12:29	01:50:40	300000	6640	25	0	0.130	**9.75**	0.045	0.07	4 195	1 938	**611**
35	Period	1806	12:29:06	28/09/2018 12:29	01:50:43	860000	6643	25	0	0.158	**11.84**	0.051	0.09	4 673	1 918	**465.6**
36	Period	1807	12:29:09	28/09/2018 12:29	01:50:46	810000	6646	25	0	0.139	**10.42**	0.047	0.07	4 608	1 775	**573**

Figure 2. Example of exported raw data from eDacq to Excel. Tidal volume and respiratory frequency values of a resting mouse from an experimental group (male, 33 days, 13.5 g) shown in blue. Corrected tidal volume by the animal weight and respiratory frequency from period values shown in darker blue.

A. Export data to an Excel file
   1. Start Excel with a blank workbook.
   2. On the **Data** tab, in the **Get External Data** group, click **From Text**.
   3. Browse your ***.csv file** (eDacq generates two files, choose the file without the period word) and click **Import**.
   4. Text import wizard will appear.
      a. Select **Delimited** option.
      b. Choose **Comma** as delimiter.
      c. In the **Column data format** section, select **General and click finish.**
   5. Data will appear in the workbook with column headings in the top row.
   6. Correct tidal volume values (ml) by the mouse weight (g).
   7. Average repose respiratory data (final 15 min values, named Period in exported Excel file) to calculate tidal volume (µl/g) and respiratory frequency (breaths/min) (Figure 2).
   8. Perform proper statistical test according to your experimental conditions.

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9. Values obtained by the eDacq software once exported in Excel file are reported in Table 1:

Table 1. Breathing values obtained by eDacq software

Name	Units	Description
Cur t	time	Current system time
Rec t	time	Recorded time (for replayed data)
Rel t	time	Relative time from start of experiment
Rel us	us (micro-s)	More accuracy for Rel t output
S	s	Number of seconds from start of experiment
N	count	Number of breaths included in the output
nRej	count	Number of breaths rejected
Period		Current period name
User		Current user name
TV	ml	Tidal Volume, volume inspired during one breath
tI	s	Inspiration time
tE	s	Expiration time
PIf	ml/s	Peak inspiratory flow
PEf	ml/s	Peak expiratory flow
f	breaths/minute	Frequency of breathing
MV	ml	Minute Volume, volume inspired in one minute
tR	s	Relaxation time,
AV	ml	Accumulated volume
EIP	s	End inspiratory pause
EEP	s	End expiratory pause
Penh	(none)	Pause enhanced
VolBal	%	Difference between inspiratory/expiratory volume

B. (Optional) Represent breathing recording

1. Initiate eDacq program.
2. Select **Experiment** option from the toolbar.
3. Press **Data source**.
4. Select **Recorded data file** option from the available options.
5. Choose the file of interest.
6. Play recording using the toolbar (**Play** button).
7. Pause at the frame time you want to represent (**Pause** button).
8. Mouse right click on **Site 1** window recording.
9. Select **Properties** and freely change them.
10. Save the file as .jpg selecting **Save Chart** from mouse right click menu (Figure 3).

Note: Remember to set up Live data source when you are ready to start a new animal recording. Recorded data file source is only to analyze previous experiments.

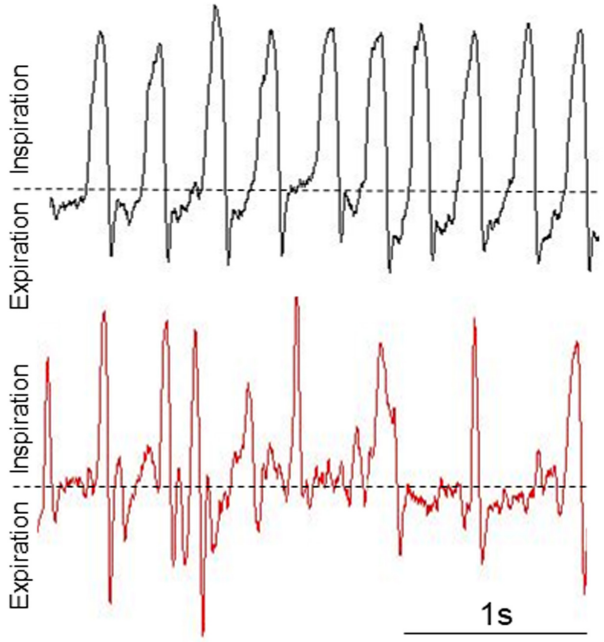

Figure 3. Representative breathing waveforms in a healthy resting animal (Top panel: Male, 62 days, 27 g) and in a resting experimental mouse presenting breathing alterations (Bottom panel: Male, 63 days, 12 g). These waveforms are not normalized by weight.

Acknowledgments

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Competing interests

Authors declare no conflict of interest.

Ethics

Original experimental data (Bolea *et al.*, 2019) was collected following the recommendations in the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use

Committee of the Seattle Children´s Research Institute and the Universitat Autònoma de Barcelona (Protocol P10127/CEEAH UAB 4155, valid until 10-2023).

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