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Noninvasive electrocardiography in the perinatal mouse

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TITLE:

Noninvasive Electrocardiography in the Perinatal Mouse

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KEYWORDS:

Congenital heart disease, developmental biology, electrocardiography, electrophysiology, heart development, noninvasive mouse ECG

SUMMARY:

Here, we present a noninvasive electrocardiography (ECG) protocol, optimized for early postnatal mice, that does not require the use of anesthetics.

ABSTRACT:

Electrocardiography (ECG) has long been relied upon as an effective and reliable method of assessing cardiovascular (and cardiopulmonary) function in both human and animal models of disease. Individual heart rate, rhythm, and regularity, combined with quantitative parameters collected from ECG, serve to assess the integrity of the cardiac conduction system as well as the integrated physiology of the cardiac cycle**.** This article provides a comprehensive description of the methods and techniques used to perform a noninvasive ECG on perinatal and neonatal mouse pups as early as the first postnatal day, without requiring the use of anesthetics. This protocol was designed to directly address a need for a standardized and repeatable method for obtaining ECG in newborn mice. From a translational perspective, this protocol proves to be entirely effective for characterization of congenital cardiopulmonary defects generated using transgenic mouse lines, and particularly for analysis of defects causing lethality at or during the first postnatal days. This protocol also aims to directly address a gap in the scientific literature to characterize and provide normative data associated with maturation of the early postnatal cardiac conduction system. This method is not limited to a specific postnatal timepoint, but rather allows for ECG data collection in neonatal mouse pups from birth to postnatal day 10 (P10), a window that is of critical importance for modeling human diseases in vivo, with particular emphasis on congenital heart disease (CHD).

INTRODUCTION:

Cardiac function can be measured in different ways, the most common of which includes the use of electrocardiography (ECG) to analyze the conduction of electric current through the heart as well as its overall cardiac cycle and function[1]. Electrocardiography continues to be a useful

diagnostic tool for identifying and characterizing cardiac anomalies in both human and animal models of disease[1,2]. Irregularities in an electrocardiogram reading can be found in abnormal cardiac development (i.e., congenital heart disease (CHD)), and can include arrhythmias manifesting as changes in heart rate (e.g., bradycardia), and rhythm (e.g., "heart blocks"), suggestive of defects in the integrity and/or function of the underlying myocardium. Changes such as these may predispose patients to life-threatening cardiac dysfunction (e.g., congestive heart failure and/or cardiac arrest) and increased mortality[3,4]. Given the high rates of mortality with severe and untreated CHD, developing a standardized and repeatable method for collecting ECG during this early postnatal period is critical.

Although we are not the first to address this problem, previous methods of collecting ECG on a mouse pups have traditionally included invasive procedures (subcutaneous needle or wire electrodes) and/or the use of anesthetics[5,6,7]. Advantages of performing noninvasive ECG analysis include minimizing pain and undo stress on the animal. While the experimenter must still be cautious about causing the pup stress, the device is designed to avoid common stressors in order to produce accurate data. In the context of evaluating cardiac function, introducing anesthesia to animals that may have cardiopulmonary abnormalities could potentially mask or even exacerbate underlying conditions. Anesthetics may affect the electrical conduction by altering depolarization and/or repolarization of the cells. Finally, the use of anesthesia can put the newborn pup at an increased risk for hypothermia, which could further confound any inherent pathology. The following protocol does not introduce any anesthetics, invasion, or pronounced discomfort to the pup. Once equipment setup is finalized, device setup and data collection involving the animal can be completed efficiently, after which the pups can be returned to their mother. Additionally, this system allows for repeat and/or serial analyses to be performed, which is ideal for experiments requiring analysis over time, introduction of pharmacological therapies, etc.

PROTOCOL:

The following protocol follows the standards of the Institutional Animal Care and Use Committee of the University of New England. Close observation of the protocol should deliver satisfactory ECG reads in all examined neonates (n > 70).

1. Device preparations

1.1 Plug the device into the USB port of a computer with the ECG software downloaded on it. The measuring device will automatically begin heating up to (37 °C/98.6 °F). The internal heating unit is contained within the measuring unit and heats only the plastic surface. The silver wire electrodes are not heated.

1.2 Allow approximately 15 min for the surface to reach the temperature. Use this time to gather and set up animals.

NOTE: The protocol may be paused at this point and the platform can remain plugged in and

heating for an extended period of time. In the absence of a self-heating electrode platform, an animal safe heating pad may also be used to keep mother and pups from becoming hypothermic.

2. Animal preparations

2.1 Collect the mother and pups and keep within the housing cage until ready to collect.

2.2 Once the measuring unit has heated to the temperature, remove the mouse pup from the cage and wipe the thorax with 70% ethanol sprayed on a wipe. Place the pup on the heated surface of the plastic.

2.3 Allow the mouse to acclimate to the surface in the dark for approximately 2-5 minutes.

3. Mouse and electrode platform setup (electrode application)

3.1 Use a metal spatula, probe, or wooden dowel to collect a small droplet of adhesive, electrical conducting gel (a quick-drying high-conductivity electrode gel commonly used for placement of rodent electrodes).

NOTE: Any nonfibrous, solid object can be used to apply the conducting gel, as long as the object will not leave behind synthetic fibers or similar material on the electrodes that could interfere with the quality of the electrical signal.

3.2 Using the spatula/dowel, gently touch the top of each of the four, flattened electrode surfaces by pressing gently down and pulling the conducting gel at an oblique angle away from the center of the electrode construct. Make sure that each individual electrode is completely covered with the gel.

CAUTION: This step is extremely important to ensure that the conductive, electrode gel does not adhere to more than a single electrode. Adhesive strands that form between electrodes can conduct charge and potentially interfere with or short out the desired electrical signal. The protocol should not be paused at this time as the gel will begin to solidify and become adherent. Make sure to set up the mouse to the platform within 5-10 minutes of applying conducting gel (or equivalent conductive electrode gel substitution).

3.3 Place the metal spatula or wooden dowel with the remainder of the gel to the side.

3.4 Place the neonatal mouse pup sternum down and prone with the head of the pup facing the outgoing USB edge of the platform. Make sure that a portion of the pup's chest is covering each of the four electrodes. Gently restrain the pup's forearms by their side while simultaneously holding down for approximately 1 min to allow the conducting gel to set.

3.5 Place rubber silicone bumpers on the right and left sides of the pup. Bumpers should secure the pup on each side and provide stability to prevent excessive movements of the mouse

but should NOT prevent all movement of the mouse. Once installed, watch the mouse for a moment and adjust bumper placement as needed.

CAUTION: Do not compress the mouse too tightly as this can interfere with respiratory mechanics and respiratory rate.

3.6 Use the dowel that was set aside to apply remaining conducting gel to the grounding tail electrode and place on the rump of the pup. Apply gentle pressure to allow the gel to set before releasing the pup.

3.7 Place the final silicon bumper on top of the rump of the mouse to hold the grounding electrode in place.

CAUTION: Do not apply excessive force while placing the final bumper as this could cause discomfort to the pup and/or displace the grounding electrode.

3.8 Grab ahold of the entire platform and gently place inside the Faraday cage.

CAUTION: Use caution and ensure the top silicone bumper does not become displaced once the Faraday cage is in place.

3.9 Prior to recording, make sure the mouse pup is not moving excessively and make sure the body and head of the mouse appears secure.

CAUTION: Make sure the mouse pup's head is able to move somewhat freely within the bumpers and is not completely snout down into the platform. The raised platform is designed to elevate the mouse thorax slightly and prevent suffocation, but this should be closely monitored.

REPRESENTATIVE RESULTS:

An ideal ECG would have a clear, prominent signal that allows all waves to be analyzed in several different time frames (**Figure 1**). The laboratory initially employed a custom application of an electromyography apparatus to produce ECGs of an unsatisfactory quality, which only allowed us to analyze basic parameters such as heart rate (**Figure S1**). This inspired work with a company to develop a novel prototype ECG device specifically for the analysis of early postnatal mouse pups.

A poor-quality reading has no discernable beats, shows clear interference, and has waves or inconsistency across the reading (**Figure 2**). To achieve the highest quality ECG, follow instructions carefully. Use caution with application of conducting gel, as it is moderately adhesive, and may require additional time to allow the mouse to accommodate to the device. By doing this, it lowers the risk of the mouse moving, there being a shorting out of electrodes, and for correct use of the device. Mice should be placed on the device so the head is facing the cords that connect the device to the USB-port and in a prone position (**Figure 3**). The mouse should be secured by rubber bumpers to hold them securely in place, with two on the side and one on the top (**Figure 3**). These bumpers should secure the mouse, but should not inhibit the mouse from

moving its head. The layout of the mouse is important for the reading, as the leads are stationary. The leads are set up so that the front two electrodes are Lead I (**Figure 3**). The rear two electrodes are Leads II and III, with the ground electrode being on the rump of the pup (**Figure 3**). Setting the mouse up in this way will allow for better results.

The program used allows for the analysis of the ECG in the program. This provides analysis of key aspects including heart rate, R-R intervals, QRS complex interval, QT interval, and PR interval. Given this ability, it was possible to establish a data set of normative values for a perinatal mouse (**Table 1**). These normative results were based off mice who were analyzed within one day after birth. It was found that an average heartbeat was 357.2 beats per minute**.** The average R-R, QRS, QT, and PR intervals were 169.1, 16.9, 45.4, and 36.3 milliseconds (ms), respectively (**Table 1**). Importantly, the setup can be used to analyze ECG patterns from neonatal mice suffering from congenital heart defects (**Figure S2**).

FIGURE AND TABLE LEGENDS:

Table 1. Representative results of ECG measurements for the average perinatal mouse pup P1, P3, P5, and P7.

Figure 1. Representative electrocardiographic reads from neonatal mice on the first (A, P1.0), third (B, P3.0), and seventh (C, P7.0) postnatal day. (**A-C**) Images represent examples of good quality ECG tracings using the 2-lead, noninvasive device, captured in a 1.5 s frame of the reading. Notable characteristics of good ECG reads include clear, discernable beats, as described collectively by the presence of consistent P-waves followed by a QRS complex and subsequent T-waves, visible in both Leads I-II of each postnatal time point. Examples also include a low signal-to-noise ratio (minimal artifact) and a discernable isoelectric line. Top ECG strip (red): Lead I; bottom ECG strip (green): Lead II.

Figure 2. Representative ECG read with complications. This image is representative of a poor-quality ECG reading using the 2-lead, noninvasive device on the first postnatal day (P1.0). The above images were captured in a 1.5 s reading frame. Poor quality ECG tracings are characterized by the absence of discernable beats (and specific cardiac cycle waveforms), along with pronounced artifact (high signal:noise ratio), and notable inconsistencies between Leads I and II from a given mouse pup. To improve this ECG, both the device and the silicone bumpers securing the pup would require repositioning within the Faraday cage. To minimize electromagnetic interference, removal of all moving devices near the apparatus would need to be carried out. The final troubleshooting measure would involve repositioning of the mouse pup on the device electrodes and/or more conductive gel would need to be (re-)applied. Top ECG strip (red): Lead I; bottom ECG strip (green): Lead II.

Figure 3. Placement of the mouse pup and limb lead electrodes for collection of early postnatal ECG. (**A**) **Left:** Anterior perspective of mouse placement on electrode platform within the Faraday cage (black). **Right:** Lateral view illustrating proper mouse placement on top of raised electrodes/platform; supportive silicone bumpers (not pictured) are placed to either side and across the top of the mouse pup within the Faraday cage. (**B**) Bipolar limb leads and electrode

placement on the neonatal mouse. Illustration depicts the point of contact for each raised electrode on the ventral thoracic surface of the mouse pup. (**B,C**) Electrode placement, chest lead directionality, and (**C**) corresponding, representative ECG tracings from a neonatal mouse pup at P1.0 (Lead I (red); Lead II (green)).

Figure 4. Representative ECG tracings of neonatal mice at multiple postnatal time points. Representative ECG reads (**top 2 traces**) and illustrated cardiac cycles (**bottom row**) from neonatal mouse pups on the first (**A**, P1.0), third (**B**, P3.0), and seventh (**C**, P7.0) postnatal day. Each image represents an exemplary ECG tracing using the 2-lead, noninvasive device, captured in a 1.5 second frame of the reading (**A-C,** Lead I (top/red); Lead II (bottom/green)). While individual waveforms do appear to undergo morphological changes with increasing age, notable and consistent characteristics include clear, discernable beats, as described collectively by the presence of consistent P-waves followed by a QRS complex and subsequent T-waves, visible in both Leads I-II of each postnatal time point.

Figure S1. Illustration of traditional limb lead electrodes for noninvasive collection of early postnatal ECG. (**A, left**) Lateral view of mouse and electrode placement within Faraday cage (box). (**B**) Traditional self-stick skin electrodes are positioned on the dorsal surface of the pup. (**A, right**) ECG-like signal may be interpreted with the use of traditional electromyography transducer to produce a minimalistic ECG tracing discernable only in Lead II (**C, bottom**). (**B-C**) Electrode placement, chest lead directionality, and corresponding, representative ECG tracing from a neonatal mouse pup at P1.0 (Lead II; purple).

Figure S2. Comparative electrocardiographic reads from littermate control pups and mutant pups with congenital heart disease on the first postnatal day (P1.0). (**A,B**) Images represent examples of good quality ECG tracings from healthy neonatal pups (**A, CONTROL**) compared to pups born with CHD (**B, CHD MUT**) at P1.0. The 2-lead, noninvasive device was used to capture ECG tracings at 10.0 (**A,B, top**) and 1.5-second intervals (**A, B, bottom**). Noticeable differences in heart rate are apparent in the CHD MUT (**B**), as visualized by the decreased number of cardiac cycles (complexes) visible in the given time frame. Comparison also reveals irregularities in the general morphology of QRS waveforms, frequency, and overall regularity of cardiac cycles in the CHD MUT (**B**) when compared to the control (**A**)**.** Lead I (red); Lead II (green)).

DISCUSSION:

The data points collected in perinatal day 1 mouse pups are slightly below the average expected values for adult mice (500-700 beats per minute).[8] There is an increase in heart rate as the mouse ages, which falls more in line for the expected values (**Table 1**). However, it is important to emphasize that neonatal values were on the lower end of this range, supporting the idea that normative values should be documented in an age-specific manner. This method is different than other electrocardiogram protocols in that there is no physical trauma to the mouse. The protocol is totally noninvasive, does not require the use of anesthesia, and is optimal for mice immediately after birth. No other electrocardiogram device allows for pups this young to be analyzed in this manner[9,10,11]. This protocol aims to establish a reliable reference method to generate normative data specific to the neonatal mouse population, but applicable to human pediatric populations.

When performing an electrocardiogram on such a small animal, it is important to be cautious with all steps. However, there are a few key steps that can change the quality of the results. The first is applying the conductive gel. If there is too much gel, there will be a higher chance for the electrodes to connect and short. If there is not enough gel, there will not be a secure connection. The best method to apply the gel is to approach the electrode from the outside corner and roll the gel over the top of the electrode. It is very important that extreme caution is taken to assure there are no threads between electrodes, which would interfere with the presence and/or quality of the electrical activity. It may be useful to take a thin tool (e.g., forceps), and run it between the electrodes to collect any stray threads which may not be apparently visible. While not formally required as part of the protocol, this extra step could serve as an additional precaution to ensure optimal conduction and minimal noise.

If the presence of noise of static causes the ECG to be unreadable (**Figure 2**), it may be useful to remove all electronic devices from the immediately (table-top) vicinity. This is especially helpful if any of the electronic devices present nearby are moving, as this movement can be picked up by the ECG recording device[12]. It is also important not to introduce any outside movements during data collection. Outside movements that could interfere with the quality of the ECG could include setting objects down on the same nearby surface, and must be avoided until after the reading is complete. In addition to outside devices, very active mouse pups may also cause electrical interference associated with excessive body movements. The likelihood of this type of musculoskeletal interference increases as the pups mature, which should be considered when selecting ages for data collection. In the event that the pup shifts from the electrodes in a way that significantly compromises the quality of the ECG reading, repositioning of the pup should be considered. Repositioning the mouse before opting to reapply the electrode gel can provide improved results in most cases and save additional time and reagents. Before repositioning the pup, select the pause button in the software. Pausing the run will stop active recording of the ECG but will continue to track time. Of note, when the recording is resumed, the ECG will appear at a later time than paused at. Slide the device platform out from the Faraday age with the mouse still positioned between the bumpers. Remove the bumpers surrounding the mouse, and gently lift the pup off of the electrodes. Reposition pup on the electrodes following the same protocol of gently holding the mouse in place for 1 min for gel to adhere (step 3.4-3.5). Try to reposition the mouse so that the electrodes are on the thorax between the upper limbs (**Figure 3**). While designed as an ideal, noninvasive method for collecting ECG in neonatal mice, one limitation associated with this protocol would be the increased mobility associated with data collection on an un-anesthetized mouse, as the mouse may also move and shift on the device which will affect the quality of the reading. While movement may be limited with positioning of silicone bumpers, this cannot be prevented without the use of sedation or anesthesia.

In the situation in which an ECG recording comes with heavy interference (**Figure 2**) despite having minimized all electrical interference, the next step that should be taken is to reposition the external wiring connecting the recording platform to the Faraday cage. It is very important that the external wiring remains properly connected to the recording platform during data acquisition. If external wiring is repositioned, be sure to reattach this wiring carefully at both

ends, until a clearer recording can be obtained. If the use of the Faraday cage provided with the device is not suitable, the device can be used in other Faraday cages.

If the recording is not clear or the mouse has moved from the electrodes, remove the mouse from the device and clean the electrodes by taking forceps and removing all the conductive gel. Because the conducting gel is water-soluble, one can also use warm water to gently remove excess gel from the skin of the pup. Reapply the gel and reposition the pup.

To obtain the best results make sure the device is properly cleaned before and after each use. The gel does dry and can be removed using forceps to pull it from the device, but the gel is water soluble, so a damp cloth can be used to clean the electrodes of the recording platform.

Older mice have been more active in the recording process, so it is important to closely monitor them as they often move from the electrodes and can even move off the device platform. While a clear read may not happen right away, with troubleshooting and repositioning, there has been success in getting usable recordings with this device (**Figure 1**). Active mice may need to be returned to their mother and reanalyzed after a break. They can also be held in the palm of a hand and gently covered to provide heat and darkness until the pup settles down.

This device is designed to collect ECG data on mouse pups from the age of birth to P10 (**Figure 4**). Pups older than P10 will likely not be able to fit into the device with the Faraday cage, an essential component to maximizing signal to noise ratios. Even at P10, positioning adjustments will likely need to be made to accommodate a larger body size into the device. Use extreme caution when moving the device into and out of the Faraday cage. Removal of the top bumper will allow the mouse to lay on the electrode platform with the surrounding Faraday cage. Given that the mice at this age are more active, they are more apt to move off the electrodes without the stabilization of the top bumper. The top bumper can also be placed in front of the pup to help discourage the pup moving off the device.

The novelty of this device and corresponding protocol include optimization for use immediately after birth, the ability of the system to accommodate a broader age range (P1-P10) and the need addressed by this method to expand the translational applications of in vivo research methods in the field of cardiovascular physiology and beyond. Although sophisticated devices utilizing echocardiography to quantify cardiac cycles in neonate mice are available[13], one great advantage of this protocol is that it allows for a relatively simple and affordable means to address basic electrophysiological parameters, which is very attractive in the current parlous scientific funding environment.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors report no conflicts of interest.

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Figure 1

Click here to access/download;Figure;Figure1.pdf

A

P1.0

B

P3.0

C

P7.0

Figure 2

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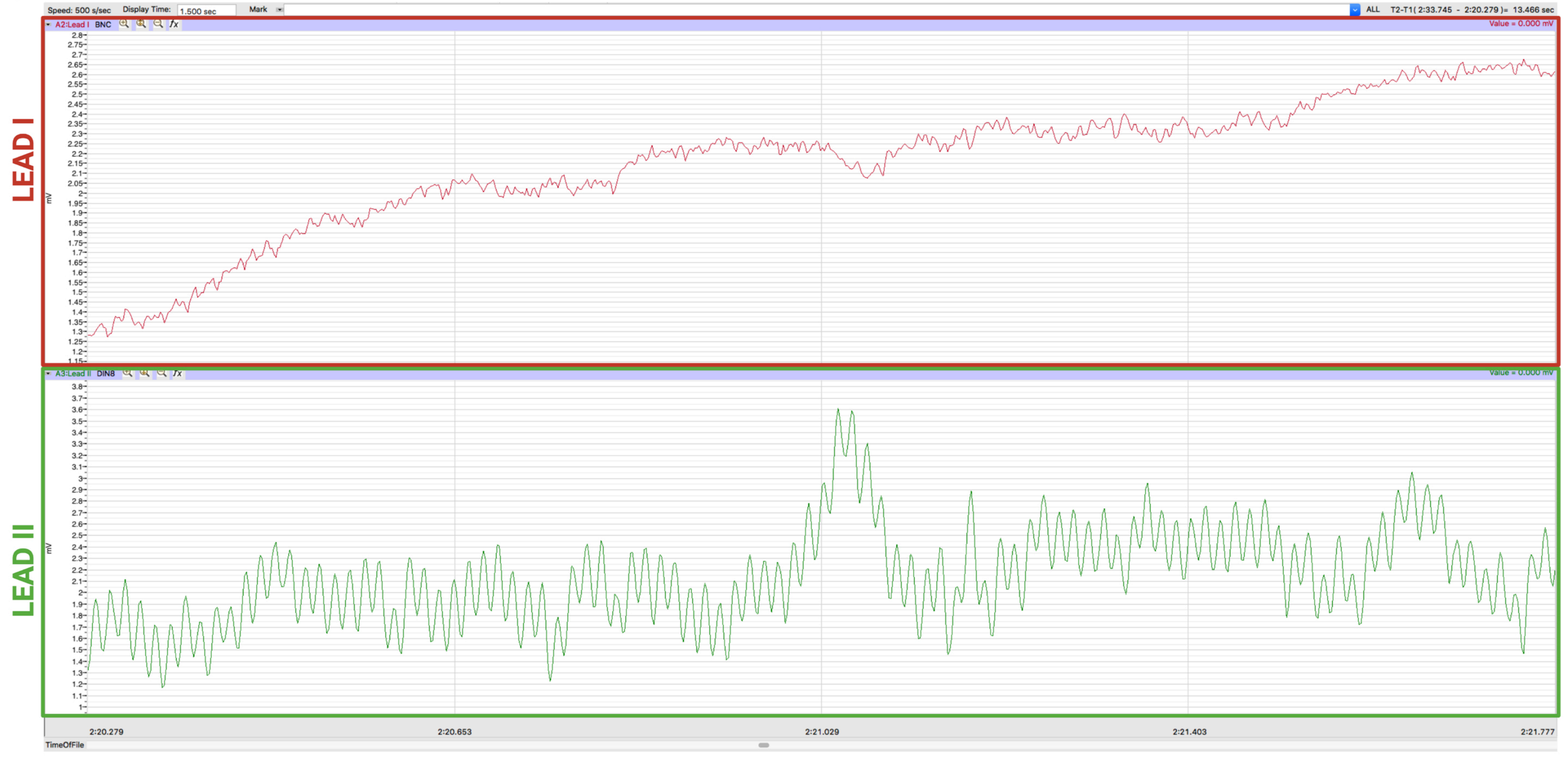

Figure S1

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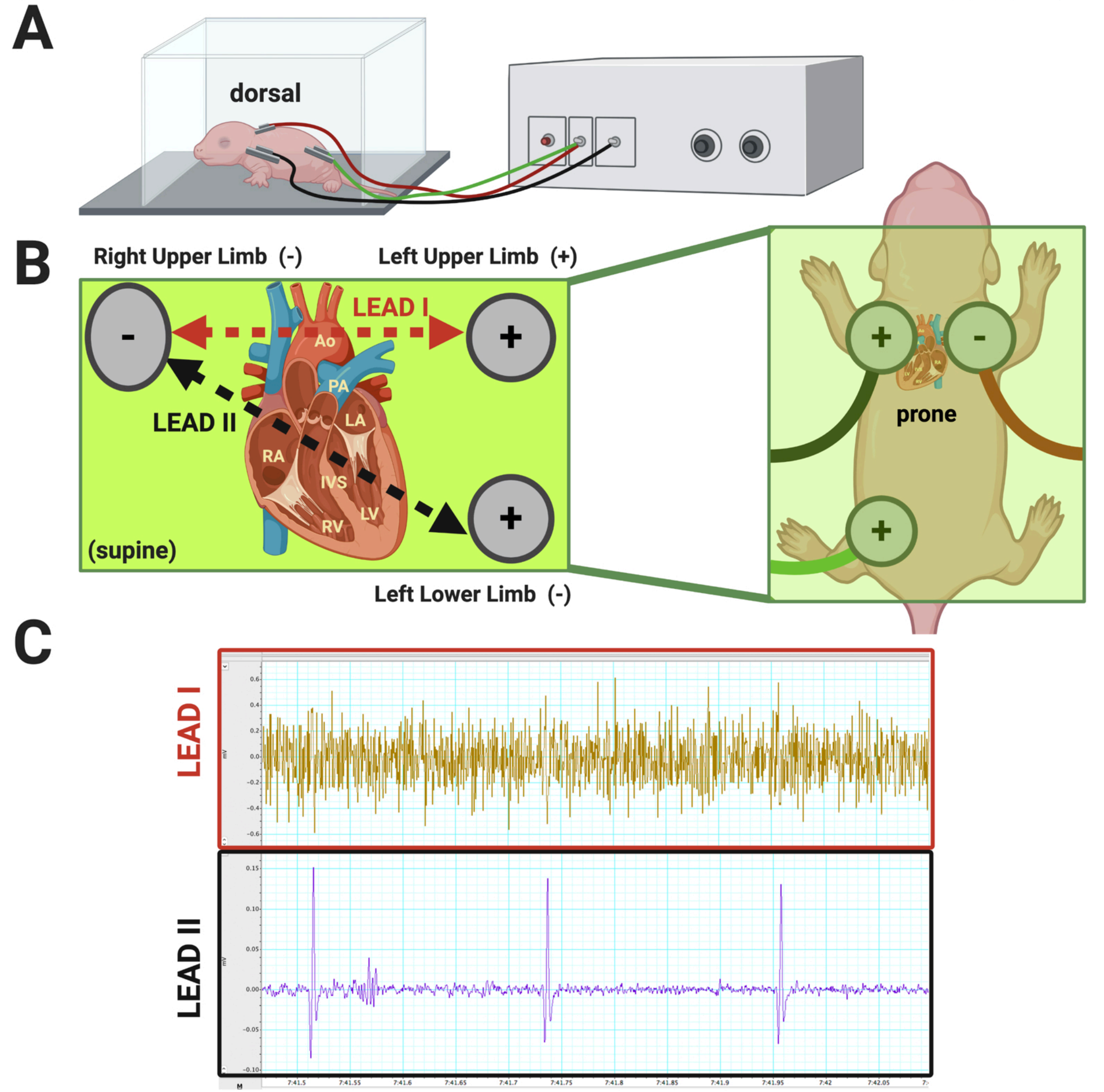

Figure S2

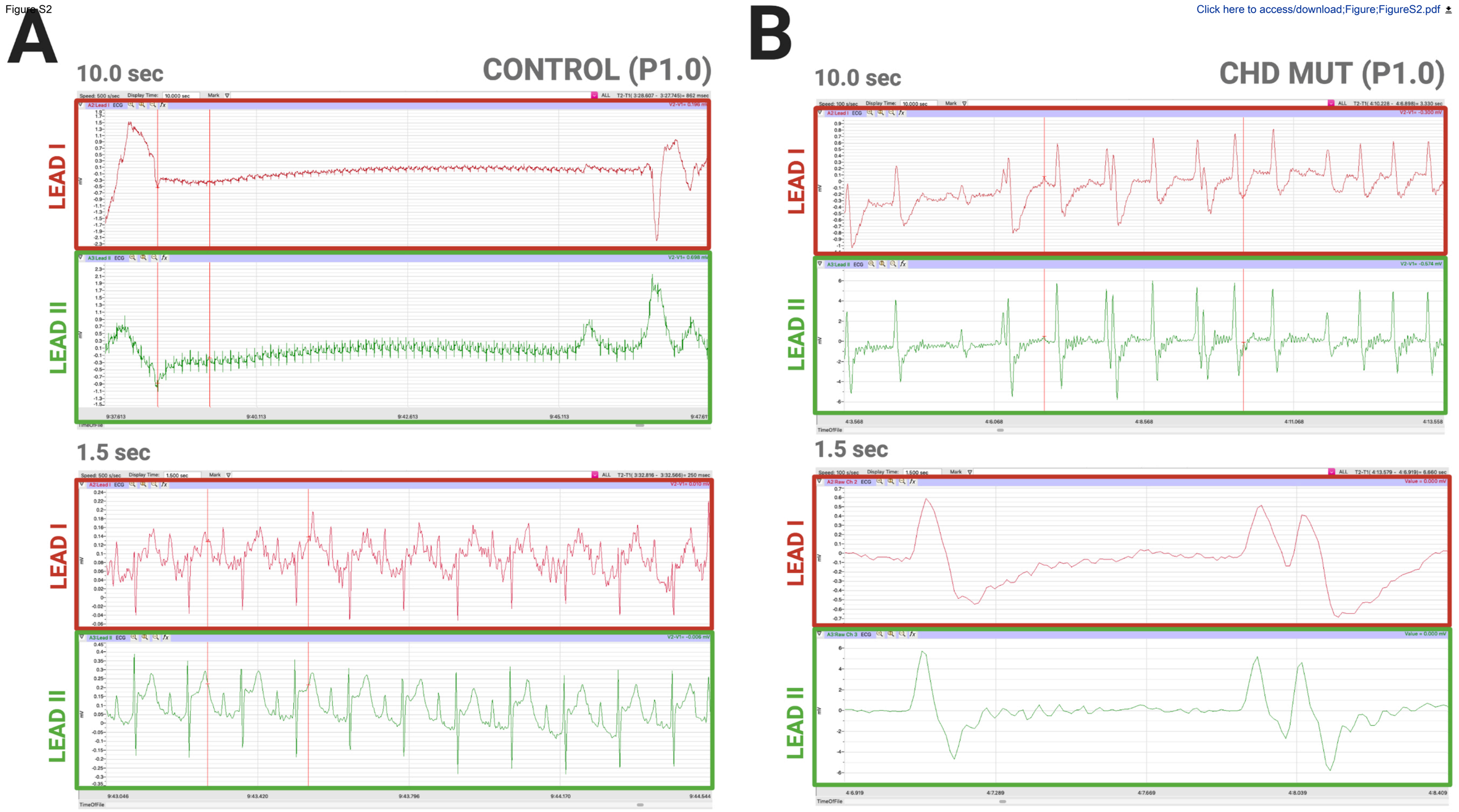

Figure 3

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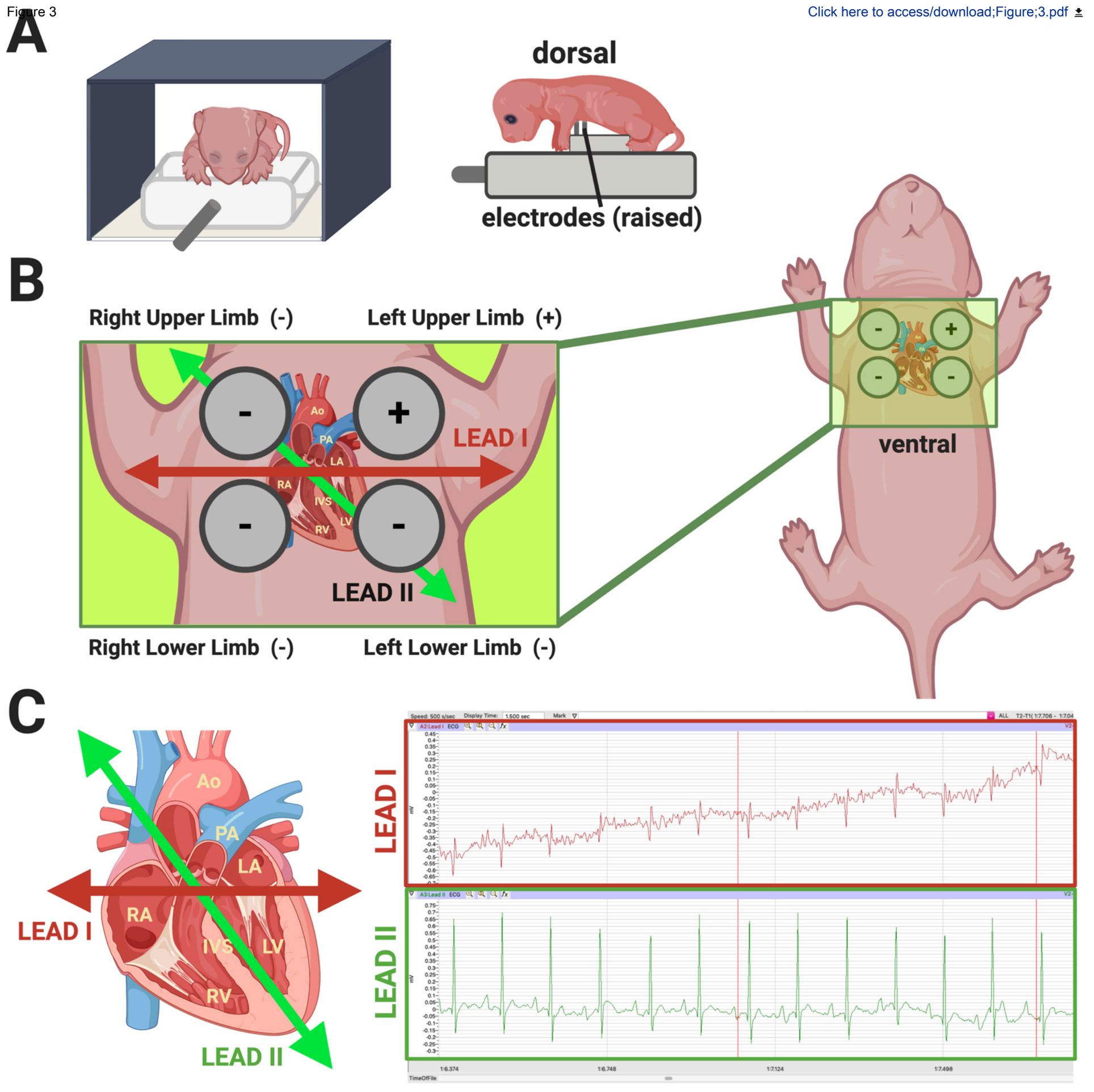

Figure 4

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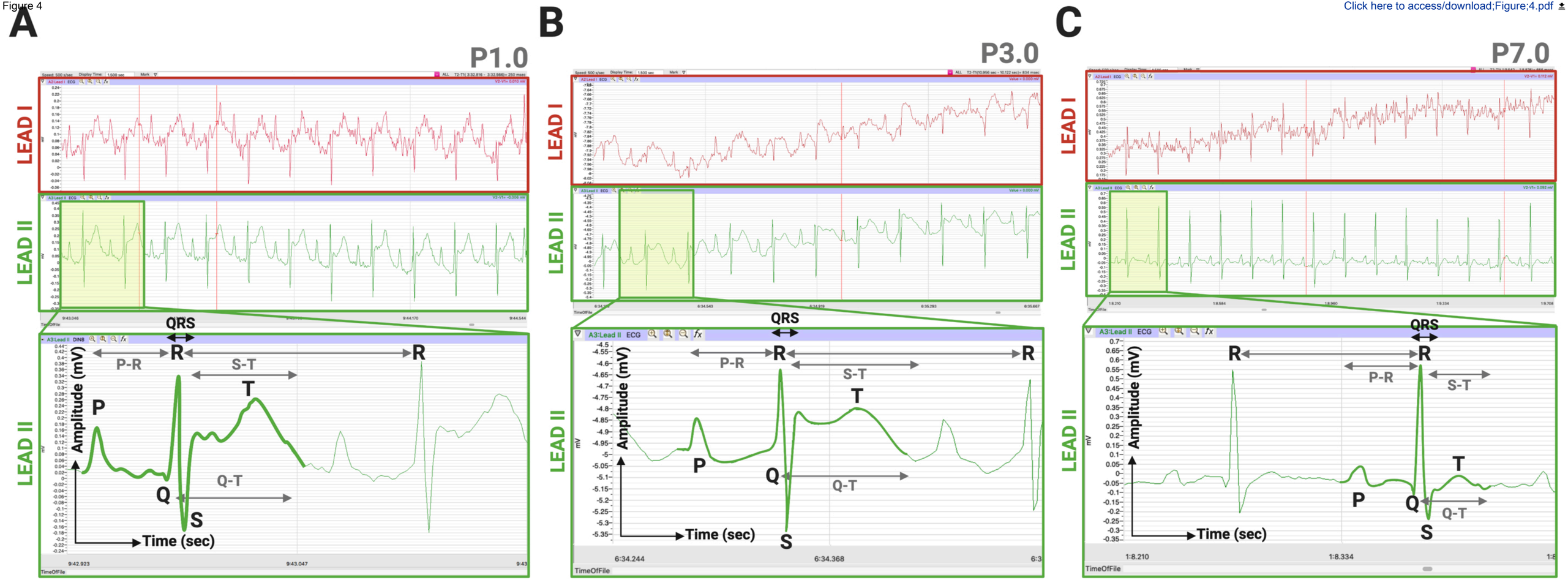

Table

Table 1

Pup Age	Ave/STDEV	Heart Rate	R-R Interval	PR Duration (ms)	QRS Duration (ms)	QT Duration (ms)	ST Duration (ms)	T Duration (ms)	P Duration (ms)
P1	Averages	357.2	169.1	36.3	16.9	45.4	16.4	18	12.8
	Standard Deviation	36.3	20	10.9	5.8	16	7.4	7.2	3.1
P3	Averages	412.4	149.2	46.4	14.5	53	22.3	16.2	14.8
	Standard Deviation	55.4	21.4	6.8	11	12.2	6.9	4.6	3.1
P5	Averages	505.5	119.2	46.7	11.7	51.3	20.8	18.8	14.2
	Standard Deviation	19.2	4.6	13.3	5.8	8.1	11.4	4.6	2.3
P7	Averages	555.3	108.7	40	9.5	43.6	20.3	13.7	14
	Standard Deviation	34.2	7	2.5	0.6	6	7.1	3.2	2.7

Table of Materials

Click here to access/download;Table of Materials;TableOfMaterials_v3.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description	
LabScribe4	iWorx	LabScribe4	Software used to record ECG	https://www.iworx.com/users/teaching.php
Neonatal Mouse ECG & Respiration System	iWorx	RS-NMECG : Neonatal Mouse ECG	ECG device	https://www.iworx.com/research/cardiac-function/rs-nmecg/
Tensive Conductive Adhesive Gel	Parker Laboratories, Inc	22-60	Tac-gel used as conductive gel for ECG	https://www.parkerlabs.com/tensive.asp

Rebuttal from the Authors

We are grateful for the sagacious commentary from the 4 reviewers. We have addressed their concerns as best we could, and we feel that the manuscript has improved tremendously as a result. Changes to the text are indicated accordingly in the revised manuscript.

A point-by-point rebuttal is shown below. The original comments from the reviewers are shown, while our authors' response (AR) is indicated by the letters "AR:".

Reviewer #1:
The authors are presenting a manuscript detailing a protocol on how to use a piece of equipment from a company (iworks). As such, my impression with the manuscript is that it is quite comparable to a manual that would come with the equipment or be available on the company website. For this to be a 'scientific publication', my recommendation is to determine the need, as is fairly well done in the abstract. Then look for or develop solutions, rather than restricting yourselves (and the readers) to a single piece of commercial equipment. Let the company do its own advertising. For example, how does the iworx thing compare to the 'LifeSpoon' from mouse specifics, inc? There appears to be some ethical advantages (see below).

AR: We agree with the comments of reviewer 1 that, on the surface, this article may have the appearance of a "user manual" for a commercially-available device. What we have not at all described in this protocol is the months of close collaborative work that took place between our laboratory and the company (iWorx, Dover, NH) that sells the device. We went through changes in every possible component of the recording platform, including the base, the chemical composition of the electrodes, the Faraday cage, the wires leading to the recording interface, and the software itself. In the end we developed and tested six different versions of the prototype device.

As far as we understand the journal's mission, it is not the purpose of the journal JoVE to detail the historical evolution of an engineering project. Instead, JoVE focuses on the step-by-step explanation of a scientific protocol, both at the written as well as at the videographic level, for the use of the general scientific audience. We are proud of our successful industrial/academic collaboration, and even more proud of the fact that we have thereby developed a protocol that is accessible to small, underfunded research laboratories that cannot afford the fancy devices, such as the Lifespoon, that require the investment of thousands of dollars.

If the editors at JoVE would like us to detail all of the iterative steps that we took in order to arrive at this working solution, then we are happy to do this. In addition, we have described the original, self-made prototype that we developed for this purpose, which delivered unsatisfactory results. Please see Fig. S1. The insufficient quality of these recordings is what prompted us to develop the device described in this article, and the improvement is readily apparent.

You write in the abstract that the protocol proves to be effective for characterizing congenital defects. Please show this.

AR: We have provided data from a mouse pup suffering from congenital heart defects, see Fig. S2.

Specific comments:
Title: "Optimizing" - what are you optimizing? There are no before and after situations.

AR: Please see first comment above.

Point 2.1: Why wipe the thorax with ethanol? Is this associated with ethical concerns in the newborn pup?

AR: The ethanol is never sprayed directly on the mouse. It is sprayed onto a Kim wipe, away from the mouse. The Kim wipe is then gently rubbed on the mouse (mentioned in step 2.1 of the protocol). This step was found to produce higher quality ECGs, but would be optional at the digression of the recorder. It was included in the protocol as it was used in every image presented. We do not believe that this represents an ethical concern, and the institutional animal care and use committee at our University is in agreement with us, as attested to by the statement at the beginning of the detailed protocol.

Point 3.1: Tac Gel appears to be an adhesive. Is restraining of the newborn pup in this way associated with ethical concerns? How is the animal released from the glue and is the skin intact?

AR: The adhesive gel is indeed mildly adhesive. The pup is able to remove itself from the device just by wiggling (which happens frequently). While the gel does hold the pup in place, it also serves an important function of being conductive. It is not the main restraint mechanism and could not be used alone on an active pup. There are also rubber bumpers that are used to safely secure the pup. These bumpers cause no harm to the pup, but instead help secure the pup on the device. It is a very frequent occurrence that the pup moves off the electrodes. This is expected as they are loosely restrained for optimal safety and comfort. The restraint methods are in place to discourage this, but in no way disallow the pup freedom of movement. The gel used is non-toxic and water soluble. The skin of the pup has never torn in the lab's use of the gel, nor does the skin show any signs of abrasion or damage after the short recording time period. We have added discussion of this topic into the troubleshooting part of the protocol.

Figures 1-3: The resolutions of these screen shots are very low. What is shown with the respiration? One long expiration? Perhaps heart rate can be read from this tracing (Figure 1), but clearly not QRS, QT or PR as is reported in Table 1. Figure 3 show that you are recording with a very low sampling frequency - 4kHz should be minimum. Lead II (not '2') appears over-filtered, but it is difficult to see at this resolution. The T waves and especially the end of the T waves are impossible to discern on these traces.

AR: We thank the author for the numerous perceptive comments. We have endeavored to record many more ECG readings from multiple timepoints, and we have discovered

that the T wave is indeed much more difficult to discern on the first postnatal day, which may represent an interesting observation regarding the maturation of the cardiac conduction system over the first postnatal week. We have included both better ECG reads at postnatal day 1, as well as multiple examples from subsequent postnatal days 3 and 5, and we believe that these represent a substantial improvement in quality. We have left out the respiration traces.

Figure 5: The authors should revisit their ECG textbook again. Lead II is not recorded between two negative electrodes. What is the difference between the 'right leg, ground' and your 'rump electrode'? Lead III is not comparable to a Z-axis, as the three leads are in the same plane. Actually, I assume lead III is calculated by the software.

AR: We thank the reviewer for this perceptive comment, and we excuse ourselves for this basic mistake. The reviewer is of courser absolutely correct, lead III is calculated by the software. In our original submission, we used the following statement: “Lead III is calculated from lead I and II and represents a lead along the Z-axis.” We have removed this statement and all references to Lead III from the article.

Reviewer #2:

Major Concerns:

1) A very critical pitfall in the method is that the ECG signals are not identical even in the adjacent cardiac cycles as shown in every displayed figures. This raises doubt about the real application of the proposed method.

AR: We have generated all of the ECG reads, taking care to ensure reliability, please see Figs 1, 3, 4 and Supp Fig. 2.

2) The authors did not provide sufficient information on how readers can obtain or prepared the electrode platform in order to use in their studies.

AR: We have indicated the provenance of the electrode platform in the Table of Materials and Reagents, and not in the text itself. This is a stipulation dictated by the Journal of Visualized Experimentation.

3) The study did not provide comparative data with other methods of ECG recording in the same age.

AR: We have provided a comparison with a device that we had used previously, see Fig. S1. The insufficient quality of these recordings is what prompted us to develop the device described in this article, and the improvement is readily apparent.

4) The study did not provide enough application of the method such as comparing results of normal pups recording with other exposed to prenatal toxicity.

AR: We have provided data from a mouse pup suffering from congenital heart defects, see Fig. S2.

Reviewer #3:

ECG is crucial in understanding cardiac physiology and pathophysiology. The manuscript highlights this well. However, it seems to convey the idea that it is sufficient to gain insight on cardiac function. Cardiac function is routinely assessed in both pre-clinical and clinical settings using echocardiography (and other imaging methods). This allows acquisition of extensive functional and structural data. I think a more balanced point of view would be useful to the readers. Interestingly, a recent publication in Ultrasound in Medicine and Biology by Castellan et al. (https://doi.org/10.1016/j.ultrasmedbio.2019.09.012) describes an echocardiography set up allowing acquisition of cardiac functional and structural data in neonatal mice by using an ECG based method. It contains heart beat measurements, amongst others, which could be used here to compare anaesthetised vs. non-anaesthetised mice. It also shows ECG traces which should probably be discussed in this article. How could those two methods complement each other?

AR: We thank the reviewer for alerting us to this interesting publication. This article has been referenced in the text, see reference number 13. Ultrasound devices, although of enormous importance to cardiovascular biology, are expensive, costing in the tens of thousands of dollars. We think that the utility of our protocol is that it offers a very low-cost alternative compared to other commercially-available devices (e.g. Lifespoon).

It would be useful to make it clearer to the un-experienced reader whether the system itself has been adapted in any way for neonatal mice. It seems that the system was already available but that no-one tried it as the manuscript stands. In other words, what exactly has been adapted by the authors to achieve their goal?

AR: We agree with the comments of both this reviewer and also reviewer 1 that, on the surface, this article may have the appearance of a “user manual” for a commercially-available device. What we have not at all described in this protocol is the months of close collaborative work that took place between our laboratory and the company (iWorx, Dover, NH) that sells the device. We went through changes in every possible component of the recording platform, including the base, the chemical composition of the electrodes, the Faraday cage, the wires leading to the recording interface, and the software itself. In the end we developed and tested six different versions of the prototype device.

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If the editors at JoVE would like us to detail all of the iterative steps that we took in order to arrive at this working solution, then we are happy to do this.

A few details would need to be added to the protocol:
-The steps following the acquisition. How are the pups removed from the instrument? How is the re-introduction to the mother done?
-In older mice, is hair removed?
-What is the success rate of acquiring a usable reading?

AR: a) We have described how pups are removed from the instrument and returned to the mother.
b) The pups are measured before they develop fur, so removal of fur is not needed.
c) The success rate has been indicated in the protocol. “Close observation of the protocol should deliver satisfactory ECG reads in all examined neonates (n > 70).” Of course, success will not be at this high rate with initial users, but we have been able to train students in this protocol over the course of a week.

Reviewer #4:

Major Concerns:

The representative results shows ECG indices obtained at one time point (P1).

- It would be beneficial for this article adding admeasurements obtained on other time points (eg. P3, P7 or P10..etc) this will ascertain the feasibility for obtaining serial measurement noninvasively, even after the pups start to become more strong, mobile and have thicker skin. Can this be accomplished without anesthesia ant P7 or P10?

AR: We thank the reviewer for this excellent suggestion. We have provided data from three separate postnatal stages, see Fig. 1 & 3. Unfortunately, at P10, the pups are too active to be able to record ECGs without some form of anesthesia and/or immobilization.

- I would suggest presenting an example (s) of abnormal ECG obtained from pups with arrhythmia or heart block. Such examples would support the applicability of this ECG devise in neonatal disease models.

AR: We have provided data from a mouse pup suffering from congenital heart defects, see Supp. Fig. 2.

Minor Concerns:
Difficult terminology/language being used for some materials/equipment being used. It would help to start the Videos/imaging part by defining or describing some of these materials.

AR: We are very eager to progress to the next step, which is the recording of the video, and we will certainly explain as many terms as we possibly can. Thank you very much for this suggestion.