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TITLE:

Acquisition of Resting-State Functional Magnetic Resonance Imaging Data in the Rat

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SUMMARY:

This protocol describes a method for obtaining stable resting-state functional magnetic resonance imaging (rs-fMRI) data from a rat using low dose isoflurane in combination with low dose dexmedetomidine.

ABSTRACT:

Resting-state functional magnetic resonance imaging (rs-fMRI) has become an increasingly popular method to study brain function in a resting, non-task state. This protocol describes a preclinical survival method for obtaining rs-fMRI data. Combining low dose isoflurane with continuous infusion of the α_2 adrenergic receptor agonist dexmedetomidine provides a robust option for stable, high-quality data acquisition while preserving brain network function. Furthermore, this procedure allows for spontaneous breathing and near-normal physiology in the rat. Additional imaging sequences can be combined with resting-state acquisition creating experimental protocols with anesthetic stability of up to 5 h using this method. This protocol describes the setup of equipment, monitoring of rat physiology during four distinct phases of anesthesia, acquisition of resting-state scans, quality assessment of data, recovery of the animal, and a brief discussion of post-processing data analysis. This protocol can be used across a wide variety of preclinical rodent models to help reveal the resulting brain network changes that occur at rest.

INTRODUCTION:

Resting-state functional magnetic resonance imaging (rs-fMRI) is a measure of the blood-oxygen-level-dependent (BOLD) signal when the brain is at rest and not engaged in any particular task. These signals can be used to measure correlations between brain regions to determine the functional connectivity within neural networks. rs-fMRI is widely used in clinical studies due to its non-invasiveness and the low amount of effort required of patients (as compared to task-based fMRI) making it optimal for diverse patient populations[1].

Technological advances have allowed rs-fMRI to be adapted for use in rodent models to uncover mechanisms underlying disease states (see reference[2] for review). Preclinical animal models, including disease or knockout models, allow a wide range of experimental manipulations not applicable in humans, and studies can also make use of post-mortem samples to further enhance experiments[2]. Nevertheless, due to the difficulty in both limiting motion and mitigating stress, MRI acquisition in rodents is traditionally performed under anesthesia. Anesthetic agents, depending on their pharmacokinetics, pharmacodynamics, and molecular targets, influence brain blood flow, brain metabolism, and potentially affect neurovascular coupling pathways.

There have been numerous efforts to develop anesthetic protocols that preserve neurovascular coupling and brain network function[3–8]. We previously reported an anesthetic regime that applied a low dose of isoflurane along with a low dose of the α_2 adrenergic receptor agonist dexmedetomidine[9]. Rats under this method of anesthesia exhibited robust BOLD responses to whisker stimulation in regions consistent with established projection pathways (ventrolateral and ventromedial thalamic nuclei, primary and secondary somatosensory cortex); large-scale resting-state brain networks, including the default mode network[10,11] and salience network[12] have also been consistently detected. Furthermore, this anesthetic protocol allows for repeated imaging on the same animal, which is important for monitoring the disease progression and the effect of experimental manipulations longitudinally.

In the present study, we detail the experimental setup, animal preparation, and physiological monitoring procedures involved. In particular, we describe the specific anesthetic phases and acquisition of scans during each phase. Data quality is assessed following each resting-state scan. A brief summary of post-scan analysis is also included in the discussion. Laboratories interested in uncovering the potential of using rs-fMRI in rats will find this protocol useful.

PROTOCOL:

All experiments were performed on a 9.4 T MRI scanner, and were approved by the Institutional Animal Care and Use Committee at Dartmouth College. Additional approval was obtained to record and show the animals used in the video and figures below.

1. Preparations before scanning

1.1. Subcutaneous infusion line

1.1.1. Partially remove a 23 G needle from its package so that the needle point remains sterile.

1.1.2. Securely hold the hub of the needle and use a razor blade to score the needle shaft where it meets the hub.

1.1.3. Clamp a needle holder around the shaft directly below the scoring and gently break the shaft from the hub.

1.1.4. Insert 1/3 of the needle shaft (blunt end) into previously sterilized PE50 line with enough line length to extend from the drug pump to the animal inside the magnet bore.

1.2. Dilution of dexmedetomidine and atipamezole.

1.2.1. Prepare a solution of diluted dexmedetomidine hydrochloride using 0.5 mL of 0.5 mg/mL stock mixed with 9.5 mL of sterile saline in a clear, sterile glass bottle (diluted concentration = 0.025 mg/mL).

1.2.2. Prepare a solution of diluted atipamezole using 0.1 mL of 5 mg/mL stock mixed with 9.9 mL of sterile saline in a clear, sterile glass bottle (diluted concentration = 0.05 mg/mL).

1.3. Scanning parameters

1.3.1. Use the parameters presented in **Table 1** to prepare scanning sequences.

2. Phase 1 anesthesia: Animal induction and preparation

2.1. Setup

2.1.1. Ensure that all equipment is on and working properly including the oxygen and air mixer, heating pad, and active scavenging system (see **Figure 1**).

2.1.2. Set the heating system's temperature set point to 37.5 °C.

2.2. Animal induction

2.2.1. Place the animal (90-day old, male Sprague Dawley rat) in the induction chamber and induce anesthesia with 2.5% isoflurane in 30% oxygen-enriched air.

NOTE: Wide range of animal ages and with both sexes can be used for this experiment.

2.2.2. Once the animal is anesthetized, remove it from the chamber, weigh the animal, and place it in the nose cone (at 2.5% isoflurane) on the heating pad in the preparation space.

2.3. Animal preparation

2.3.1. Apply ophthalmic lubricating ointment to each eye to prevent drying.

2.3.2. Confirm the depth of anesthesia by a lack of toe pinch response.

2.3.3. Use clippers to shave a 2" by 2" square area on the lower lumbar region of the animal's back (i.e., directly above the tail).

2.3.4. Administer 0.015 mg/kg of the dexmedetomidine solution with an intraperitoneal (i.p.) injection (e.g., a 300 g rat would receive 0.18 mL) into the lower right quadrant of the abdomen using a 25 G needle.

2.3.5. Switch isoflurane flow from the preparation space to the animal cradle.

2.3.6. Move the animal into the animal cradle. Place the rat's front teeth securely over and into the bite bar. Push the nose cone over the nose to ensure a tight fit.

NOTE: If the nose cone does not cover the lower jaw, use a paraffin film to gently hold the jaw closed while also sealing around the nose cone.

2.3.7. Position the respiration pad under the rat's abdomen below the rib cage and re-position it until the respiration waveform shows a deep trough centered on each breath (see respiration waveform in **Figure 2**).

2.3.8. Monitor the animal's breathing using the physiology monitoring software. Move to the next phase of anesthesia when respiration is less than 40 breaths/min (bpm; approximately 5 min after dexmedetomidine injection.

3. Phase 2 anesthesia: Animal setup

3.1. Insert ear bars into the ear canal to stabilize the rat's head in the animal cradle. Once positioned, pull forward on the bite bar and confirm the head does not move. Re-adjust the nose cone and paraffin film as needed (see **Figure 3a**).

3.2. Insert the temperature probe into a pre-lubricated, disposable probe cover. Gently insert the temperature probe approximately ½" into the rectum, and tape it to the base of the tail with medical tape.

3.3. Place the pulse oximeter clip onto the metatarsal area of the hind foot, ensuring the light source is on the bottom of the foot (palm).

NOTE: Rotation of the clip can affect the signal; thus, creating a holder to keep the paw and clip upright will lead to greater stability. Also note that until the rat is at normal body temperature, the oxygen saturation may be low (<95%).

3.4. Use the rat's weight to calculate the infusion rate to eject 0.015 mg/kg/h of dexmedetomidine (a 300 g rat receives 0.18 mL/h).

3.5. Set the drug pump to eject the calculated infusion rate.

3.6. Fill a 3 mL syringe with the sterile, diluted dexmedetomidine solution and insert the tip of the needle into the open end of the sterilized infusion line (extending from the drug pump to the animal cradle with the subcutaneous needle previously attached). Fill the line and secure the syringe in the syringe holder of the drug pump.

3.7. Move the pusher block forward until it touches the plunger, and the drug is expelled at the needle, ensuring the infusion line is completely filled.

3.8. Using an alcohol wipe, clean the shaved area to remove any stray hair.

3.9. Pinch the skin approximately two finger widths above the base of the tail. Insert 1/3 of the infusion line needle into the tented skin.

3.10. Secure the needle to the skin with a 3" piece of wide medical tape. Place a second piece of wide medical tape over the first, across the rat, and attached to both sides of the animal cradle (see **Figure 4**).

NOTE: It is critically important that the ferromagnetic needle is well secured to prevent movement during the scan.

3.11. Begin the infusion of subcutaneous dexmedetomidine.

3.12. Place a piece of gauze on the bridge of the rat's nose to create a level surface for the coil. Use paper tape, which does not interfere with the MRI signal, to secure the coil to the rat's head, centering it over the brain (see **Figure 3b,c**).

3.13. Secure all lines and cables within the animal cradle with lab tape and check whether all the physiology signals are stable (see **Figure 2**).

3.14. Place paper towels over the animal, securing them to the animal cradle with laboratory tape. If using an air heating system, wrap a plastic sheet around the entire cradle to contain the warm air.

3.15. Move the animal into the bore and tune the magnet.

4. Phase 3 anesthesia: Anatomical scan acquisition

4.1. Reduce isoflurane to 1.5%, resulting in a steady increase in respiration to approximately 45–50 bpm. Remain at this level for the duration of the anatomical scanning.

4.2. Use the FLASH localizer scan to ensure the brain is aligned with the magnet isocenter (**Figure 5a**). Reposition the animal and repeat if necessary.

4.3. Run the higher resolution RARE localizer scan and use this scan output to align 15 sagittal slices centered across the brain (left to right, **Figure 5b**).

4.4. Using the middle sagittal slice, align the center axial slice to the decussation of the anterior commissure, which appears as a dark spot (**Figure 5c**). Note the slice offset to use later in the resting-state scans.

4.5. Acquire 23 slices using both the FLASH and RARE axial protocols to aid in registration to a common space during post-scan analysis.

4.6. Shim across the whole brain using the PRESS sequence.

5. Phase 4: Resting-state scan acquisition

5.1. After completing anatomical scans, reduce isoflurane to 0.5% to 0.75%, adjusting so that the animal's respiration is 60–65 breaths per minute. Remain at this level for at least 10 min before beginning resting-state scanning to ensure stability.

5.2. When physiology is stable (respiration range is 60–75 bpm with no gasping or irregularities, core body temperature is 37.5 ± 1.0 °C, and oxygen saturation is 95% or greater), acquire a 15 slice EPI scan using the same slice offset as the anatomical axial series.

5.3. After each resting-state scan is complete, check the quality using an independent component analysis (ICA) to decompose the data into spatial and temporal components.

5.4. Obtain at least three high-quality resting-state scans.

6. Post-scan recovery

6.1. When scanning is complete, increase isoflurane to 2% and stop the subcutaneous dexmedetomidine infusion.

6.2. Remove the animal cradle from the magnet bore, unwrap the animal, and remove ear bars, temperature probe, pulse oximeter clip, and the dexmedetomidine needle.

6.3. Inject 0.015 mg/kg of the diluted atipamezole solution into the rat's hind leg muscle using a 1 mL syringe with a 25 G needle (i.e., a 300 g rat would receive 0.09 mL).

6.4. Place the rat back in the home cage on top of a heating pad and monitor until the animal is ambulatory.

REPRESENTATIVE RESULTS:

Following each resting-state scan, stability is assessed using an independent component analysis (ICA; example script included in **Supplementary Files**). **Figure 6** shows examples of component outputs from resting-state scans. **Figure 6a** shows a signal component from a scan with high stability. Note that spatially, the component has high regionality. Within the time course below the spatial component, the signal is stable and not predictable, indicative of true brain activity. The power spectrum at the bottom shows predominantly low frequencies. **Figure 6b** shows a component from the same scan as **Figure 6a** that represents noise. Note the non-regionality in the spatial component, high-frequency time course, and high frequency peak in the power spectrum. Finally, **Figure 6c** shows a component from a scan with unstable anesthesia. The time course is variable and irregular. When this occurs, improvements are needed to the anesthetic protocol, commonly to the sealing of the nose cone and the scavenging of waste gases.

FIGURE AND TABLE LEGENDS:

Figure 1: Preparation space and MRI animal cradle. a) Preparation space. The vacuum scavenges waste gases from both the induction chamber and the nose cone at the animal cradle. The heating pad helps to maintain animal temperature during both Phase 1 and recovery. b) MRI animal cradle. The top indicates components of the animal setup in Phase 2. The bottom shows a rat fully set-up and ready for scanning.

Figure 2: Physiologic scan output. Oxygen saturation (PulseOx, 96%), heart rate (325 BPM [beats per minute]), respiration rate (61 breaths/min), and core body temperature (T1, 37.5 °C) are constantly monitored throughout the scanning session.

Figure 3: Nose cone and coil placement. (**a**) Close up view of the nose cone sealed around the animal's nose and lower jaw. (**b**) Overhead view of the alignment of surface coil to the brain. (**c**) Side view of coil alignment with the midpoint of the animal's eye.

Figure 4: Subcutaneous dexmedetomidine infusion line and needle placement. (**a**) Needle insertion into the lower lumbar region of the animal's back. (**b**) Tape securing the needle to the animal's skin. (**c**) Tape across the animal cradle to prevent movement of the ferromagnetic needle.

Figure 5: Anatomical scan alignment. (**a**) Localizer scan to align the animal's brain to the magnet isocenter, noted with crosshairs. (**b**) Sagittal slices aligned across the brain from left to right. (**c**) Alignment to the decussation of the anterior commissure, indicated by the white arrow.

Figure 6: Quality assessment using independent component analysis. (**a**) Signal component during steady anesthesia. (**b**) Noise component during steady anesthesia. (**c**) Unsteady anesthesia.

Table 1: Reference table of scan parameters. Parameters for the sequences outlined in the

protocol. FLASH = Fast Low Angle Shot, RARE = Rapid Acquisition with Relaxation Enhancement, PRESS = Point RESolved Spectroscopy, EPI = Echo Planar Imaging.

Supplementary Files: Example script for ICA quality assessment.

DISCUSSION:

Stability of the animal, both physically and physiologically, is key to obtaining high-quality resting-state data. This protocol achieves stability by moving through four distinct phases of anesthesia. It is imperative that the animal has met the set physiological thresholds before moving to the next phase of anesthesia; since this method relies on physiological autoregulatory mechanisms, individual animals may require slightly different amounts of time at each anesthesia phase. It is our experience that taking more time at each phase is more efficient than hurrying through earlier stages without giving the rat's physiology sufficient time to settle. The key components that allow for stability are the fit of the nose cone and proper waste gas scavenging.

A properly sealed nose cone and scavenging allow the animal to remain stable with regularly spaced breathing and steady oxygen saturation levels. If gasping, irregular spacing, holding of the breath, or decreasing oxygen saturation levels occur, one should work to improve the nose cone sealing and scavenging. The nose cone should fit closely but should not push into the bridge of the nose. A custom nose cone may need to be fabricated. The original nose cone from our manufacturer had an air outtake valve that was too small, so a falcon tube was fitted with a larger sealed vacuum line closer to the animal. This resulted in better clearance of expired CO_2 and steady oxygen saturation. As mentioned in the protocol, paraffin film may be wrapped around the lower jaw and edge of the nose cone, but if wrapped too tightly, it can restrict breathing and lead to instability. Additionally, improper placement of ear bars and bite bar not only affect the necessary stability of the head for imaging but can also affect breathing; continued blinking or audible noise from the animal is a likely indication of improper ear bar placement. The front teeth should sit securely on the bite bar and be pulled forward after ear bar placement to ensure a tight fit. The rat's tongue may need to be pulled forward if it sits too far back in the mouth and restricts proper breathing.

As each system is unique, fine tuning the vacuum level is required to achieve optimal scavenging. As a practical guide, it should be possible to feel a small amount of suction either by placing a finger over the vacuum line opening inside the nose cone, or by sealing the entire nose cone opening with the palm. Matching flow rate for anesthesia input (0.8 L/min was used here) is a good starting point. Oxygen saturation in the animal should remain above 95% throughout the scan. If oxygen saturation shows a decreasing trend, this may be an indication that CO_2 is building up in the nose cone and scavenging needs to be increased. Another possibility is that the pressure of the pulse oximeter clip on the foot needs to be adjusted, either loosened to improve blood flow or tightened to ensure a strong, stable signal. If respiration of the animal is higher than the thresholds outlined, this may indicate that scavenging is set too high and is removing too much isoflurane. In rare circumstances, it may be necessary to increase the dose of subcutaneous dexmedetomidine to 0.02 mg/kg/hr, but we have found that 0.015 mg/kg has worked well across a wide range of rat ages and both sexes, and is supported in pharmacological studies[4].

The scan duration necessary for fMRI activation is a function of effect size, spatial signal-to-noise ratio (SNR) and temporal SNR, as shown previously by Murphy et al.[13]. The use of a small surface coil (2 cm) and high magnetic field (9.4 T) substantially enhances SNR and BOLD sensitivity. With our imaging setup, we have found that a single 6 min scan is sufficient to detect a robust resting-state functional connectivity network, consistent with our previous report[10]. Nevertheless, we typically repeat the scan 3 to 4 times, and average the results to derive functional brain networks for individual animals. Alternatively, one can scan a single time with a longer duration (10 min or more) to derive functional connectivity networks[14].

After collecting high quality rs-fMRI using this protocol, preprocess the data as has been previously published[15,16]. With the use of both ear bars and a bite bar, motion artifacts in the fMRI time course are minimal, and the use of motion correction has not had a noticeable effect on our data. Individual resting-state EPI scans need to be skull-stripped and registered to a common space (we use a single representative rat brain)[16,17]. Remove the beginning volumes from each EPI so those included are all acquired when the magnet is at steady-state (we remove 5 time points). Denoise individual scans (see **Representative Results** for examples of signal and noise components). Apply slice timing correction, as well as linear and quadratic trend removal, band pass filtering (0.005–0.1 Hz) and spatial smoothing (0.6 mm FWHM [full width at half maximum]). Additionally, remove the average signal time course from the white matter and ventricles through linear regression. After these standard preprocessing steps, further group level analysis can be performed including seed-based functional connectivity[11,15,18–22], independent components analyses[10,20,22], and modularity analyses[12,19].

There are two main advantages of the current protocol: 1) it allows for spontaneous brain activity; and 2) it keeps the animal at near-normal physiology. Alternative anesthetic methods (such as propofol[21], α-chloralose[15], and pancuronium bromide in combination with another anesthetic[21,23]) have also been used to acquire resting-state data. However, using a combination of low dose isoflurane with low dose dexmedetomidine, as described in this protocol, has been shown to only minimally disrupt brain network functions while also providing the physiologic stability needed to obtain quality resting-state functional connectivity data[9,10,18,24]. Furthermore, BOLD responses from somatosensory stimulation[9] and mechanical whisker deflection[11] can be seen at or after a period of 90 min when using this protocol, suggesting a consistent arousal level. Interestingly, using dexmedetomidine in isolation can elicit epileptic activity; however, this activity was abolished with supplemented isoflurane[8]. Another advantage to the current protocol is that it eliminates the need for artificial ventilation. Although mechanical ventilation may lead to a narrower range of partial carbon dioxide and oxygen saturation across animals, in longitudinal studies, maintaining physiological parameters without the need for intubation may result in fewer complications and unwanted side effects.

Interest in resting-state fMRI has grown considerably in the past 10 years, and with it a need to acquire high-quality, preclinical resting-state scans from rodents. This survival protocol achieves stable anesthesia for up to 5 h with near-normal physiology during resting-state acquisition. As the protocol is highly stable, additional sequences (structural, stimulation, pharmacological MRI,

etc.) can easily be added to achieve the desired experimental design. The combination of low-dose isoflurane with dexmedetomidine utilized in this protocol allows for a wide variety of preclinical studies for investigators interested in studying the rodent brain in its resting state.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1 Revised

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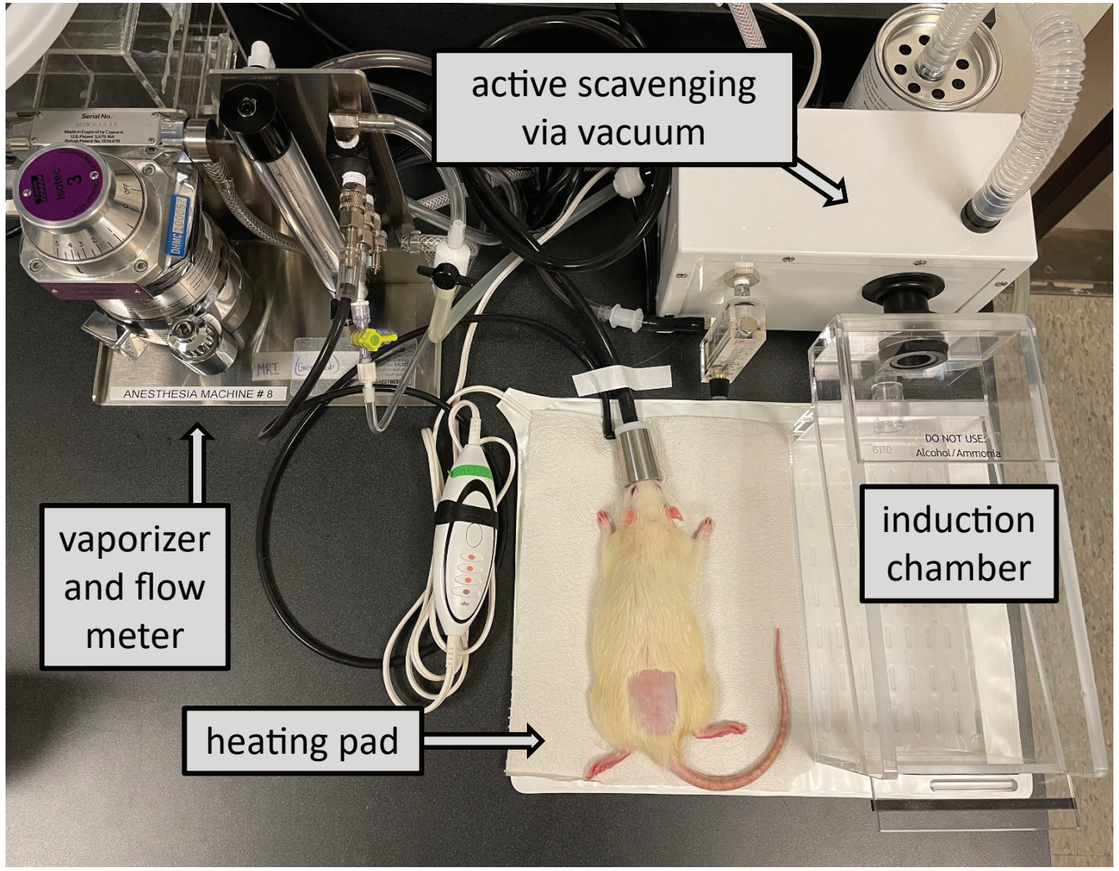
a.



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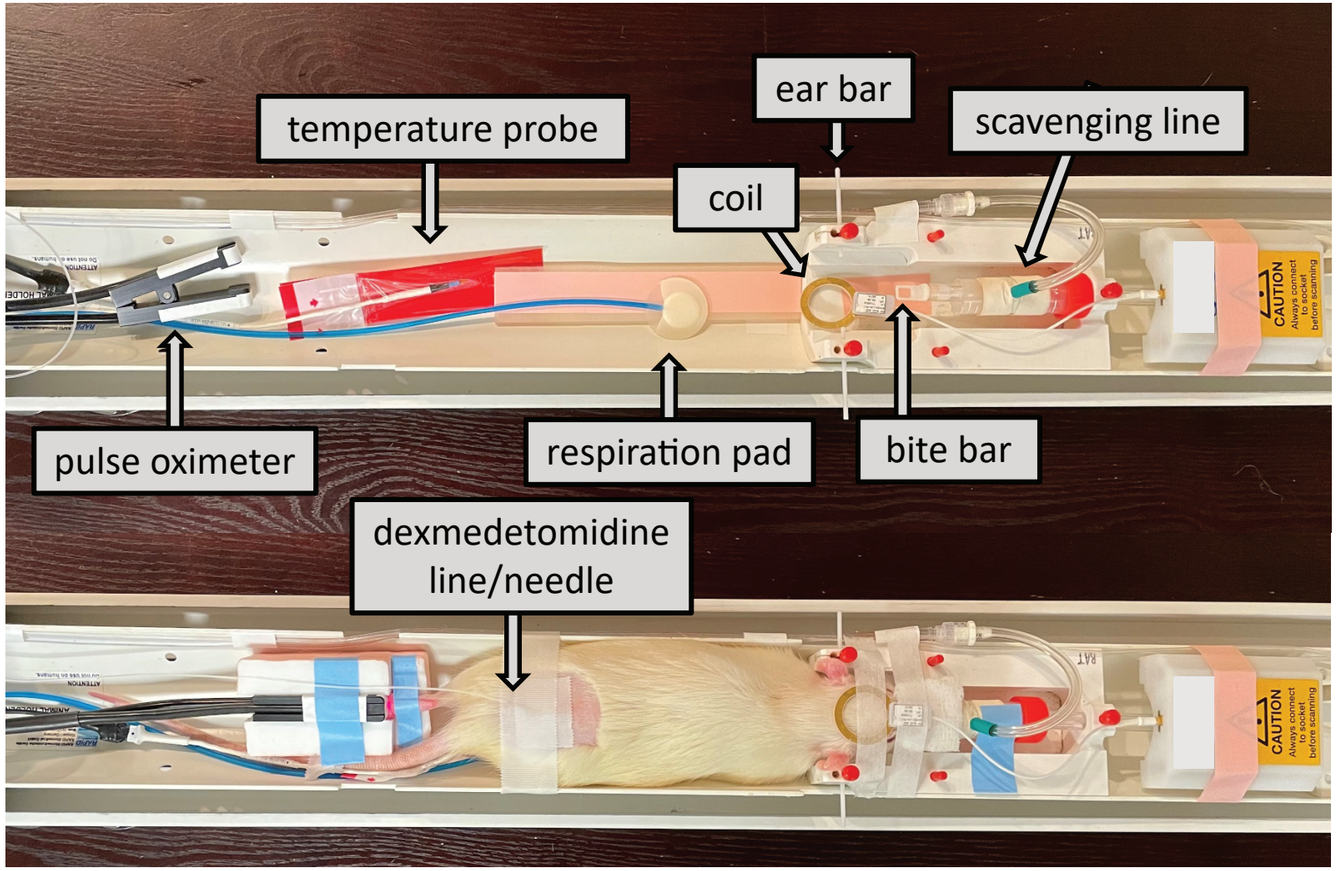

Figure 2 Revised

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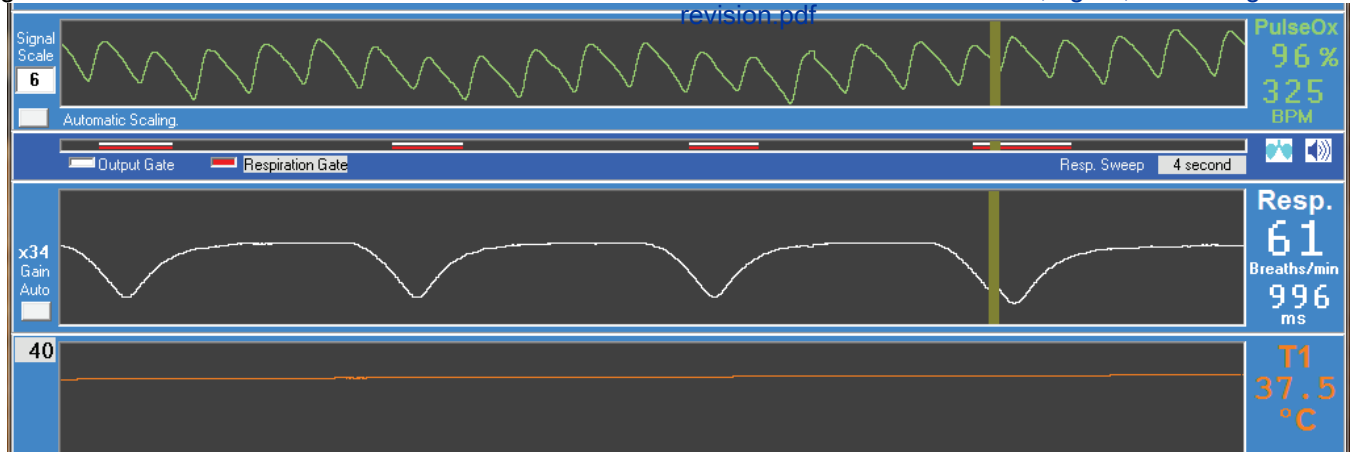

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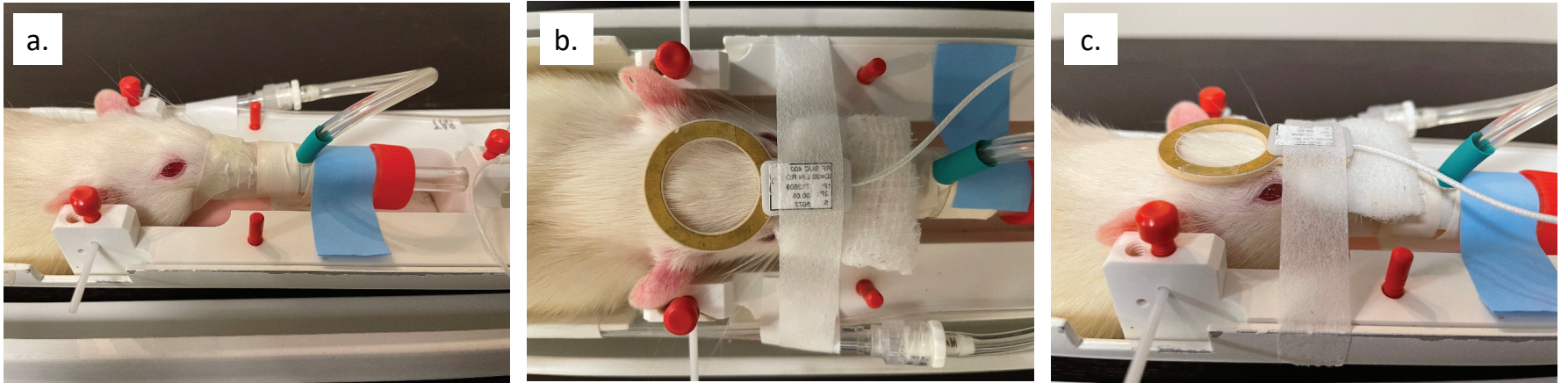

Figure 4 Revised

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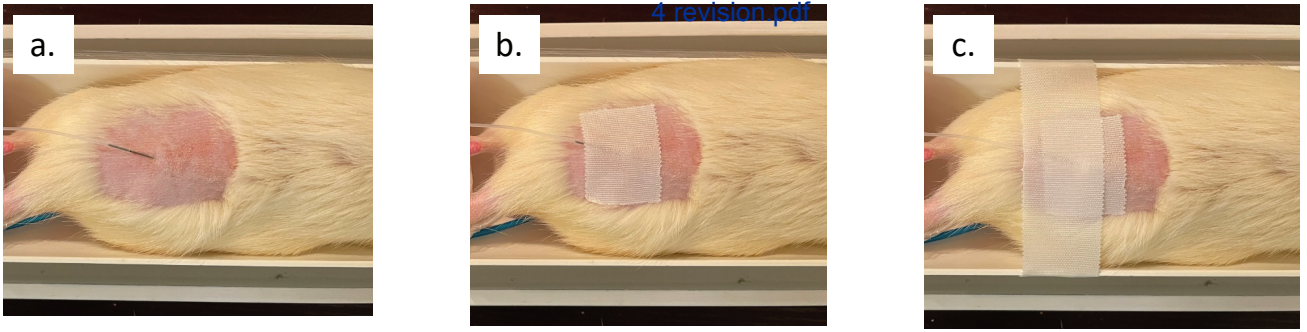


Figure 5 Revised

Click here to access/download:Figure:JOVE - Figure 5 revision.pdf

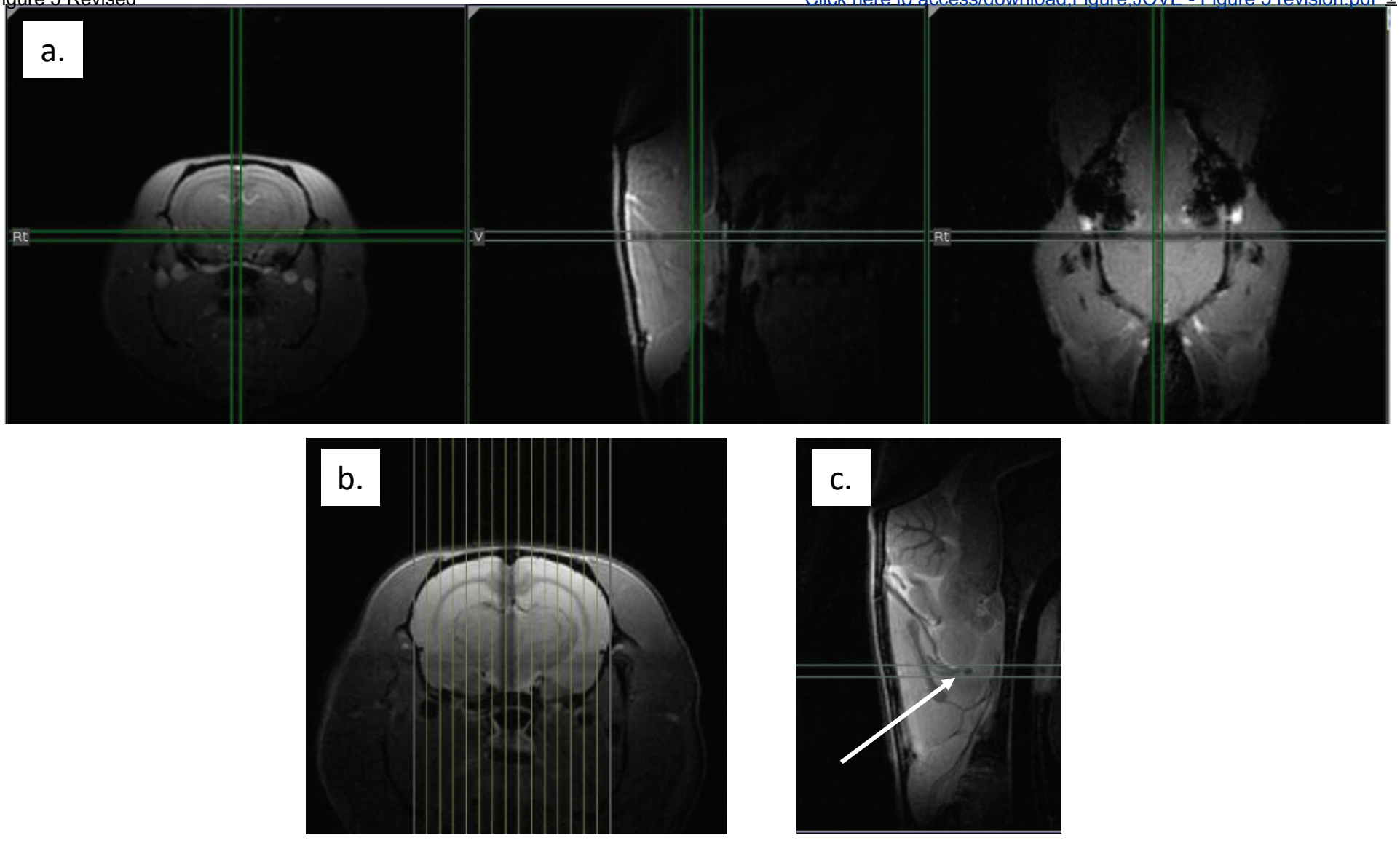
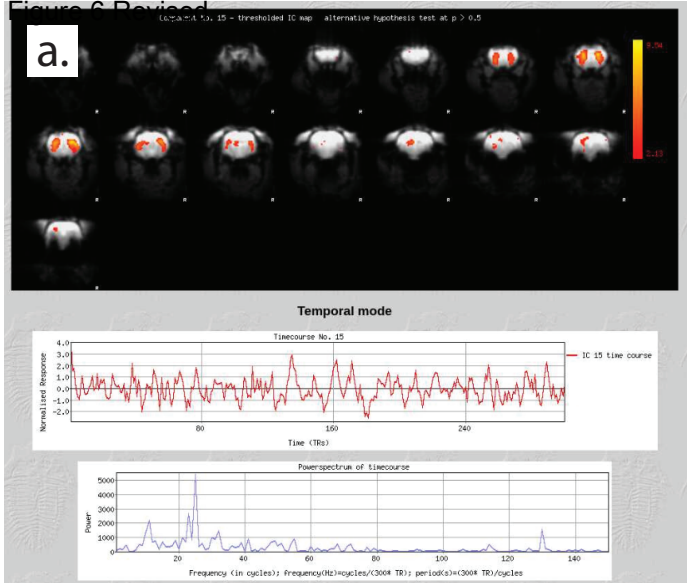
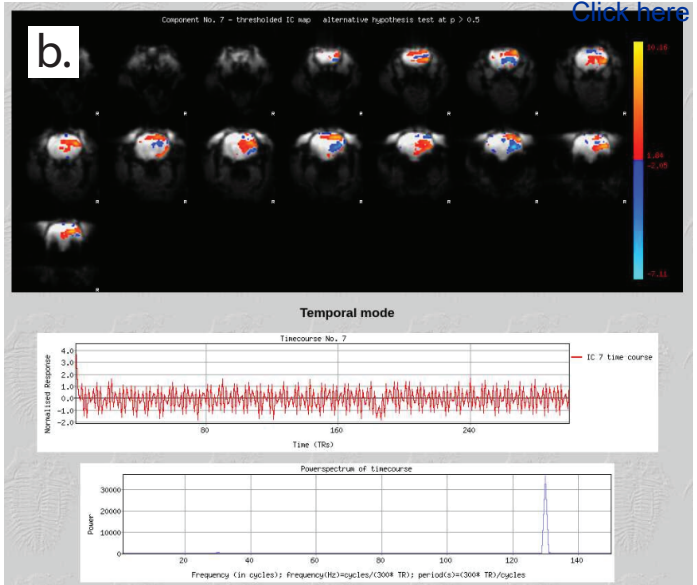
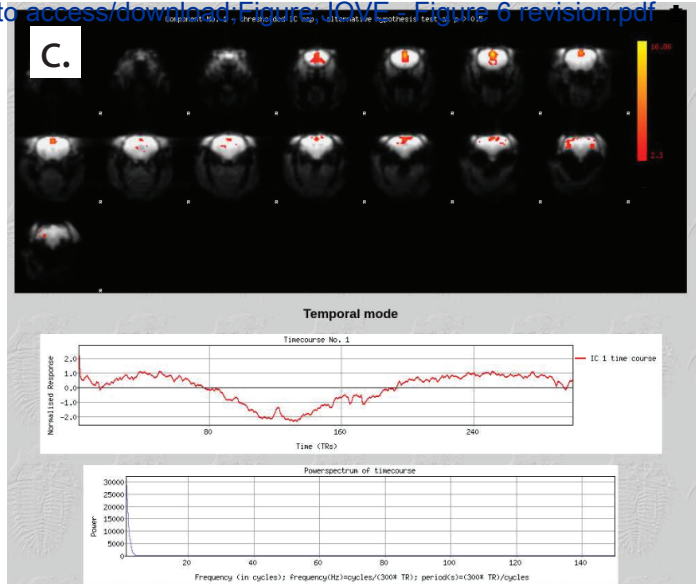


Figure 6 Revised







Scan	Sequence	Orientation	FOV (mm x mm)	Matrix	Slices	Slice Thickness (mm)	TE (ms)	TR (ms)	Averages
Localizer	FLASH	All planes	50	256	1/dir	1	2.5	100	1
Localizer	RARE	All planes	35	192	1/dir	0.75	28	2500	1
Anat	RARE	Sagittal	35	192	15	1	28	2500	1
Anat	FLASH	Axial	35	192	23	1	5	250	2
Anat	RARE	Axial	35	192	23	1	28	2500	4
Shim	PRESS	All planes					16.223	2500	1
Resting-State	EPI	Axial	35	64	15	1	15	1200	1

Echo Spacing (ms)	Rare Factor	Repetitions	Scan Time
		1	12.8 s
7	8	1	1 min
7	8	1	1 min
		1	1 min 36 s
7	8	1	4 min
		1	2.5 s
		300	6 min each

Editorial and production comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have spell checked the document and corrected grammar.

2. Please define all abbreviations during the first-time use.

We have checked all abbreviations.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: 9.4 T Agilent MRI scanner, Bruker console, Paravision 6.0.1 platform, Model 1025 Monitoring & Gating System, Small Animal Instruments, Inc. Pulse Oximeter Module, Small Animal Instruments, Inc., Model 3500CP-G, Sechrist, SAII system, model MT0105-20, Bruker, MELODIC from FSL, AFNI (Analysis of Functional Neuroimaging) software, etc.

We have removed these commercial names from the manuscript.

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please do not start from zero and refrain from using bullets, alphabets, or dashes.

We have adjusted the numbering to begin at 1 and proceed as indicated.

5. 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.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol.

Any text that cannot be written in the imperative tense may be added as a "Note."

We have rewritten the protocol section in the imperative tense, removed the phrases mentioned and added notes as needed.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We have simplified the protocol as described.

7. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion. All actions must be described in complete sentences and should follow the order.

We have moved the discussion about the protocol to the discussion section.

8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

We have added details to describe the steps as needed.

9. Some of the subheadings can be removed and the steps can be made crisper.

We have removed many of the subheadings.

10. Please include strain, sex, age of the animal used for the study.

We have included these details.

11. 0.2: Amount and concentration of Isoflurane used? How do you check the depth of anesthesia?

We have included the amount and concentration of anesthesia as well as details about how to check the depth of anesthesia (lack of toe pinch response).

12. 1.3.5: How do you check the respiration rate?

We have included that the respiration rate can be read from the physiology monitoring software.

13. Line 251: Please check if the table is correctly referenced-Is this table 2 instead?

Yes, the correction has been made.

14. Please ensure the results are described in the context of the presented technique. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

The results have been described to show how experimental quality can be determined from the ICA components. Successful and suboptimal experiments have been included.

15. Table 1: This can instead be combined with the table of materials.

This table has been combined with the materials list.

16. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have updated the discussion to include these components.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.

We have increased the homogeneity between the video and written manuscript. All figures from the video (except the overview figure) and also contained in the manuscript. Figures 1, 3, 4, and 5 in the manuscript are shown or demonstrated in video segments. Figures 2 and 6 in the manuscript are shown in the video.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol.

The narration has been re-recorded to read word-for-word the steps of the protocol, except when making clear what occurs that has not been videotaped.

3. 1:01: Please remove commercial terms from the video and use generic term instead. JoVE cannot have commercial terms both in the video and text.

We have removed commercial terms.

4. Please ensure that the chapter titles are same both in the video and in the text.

All chapter titles have been checked to be the same in the video and text.

5. 1:38 How do you check the depth of anesthesia?

We have recorded video of checking for a toe pinch response before beginning the procedure and included it in the audio.

6. 6.29: Please do not show the commercial term in this shot.

We have removed the commercial term and re-shot this segment.

7. Please include a section title card “conclusion” for the conclusion section.

We have included a Conclusion title card.

8. Title Cards:
• Please capitalize the first letter of every important word in your title.
• 10:28 to 11:27 The use of the title card at the end for the conclusion is somewhat uninteresting. Consider adding some video or image clips to pair with what is being explained in the narration.
• Please add chapter Card "Conclusion"
• Please recompose the title cards to match the video's native resolution. The title cards looks lower resolution compared to the video's 1080 pixel resolution.
• Please remove the motion/animation on the chapter title cards.

We have capitalized the title.

The title cards have been redone to match the video’s resolution and animations have been removed.

9. Video Editing Content:
• Please ensure that the Narration should start After the Main Title Card i.e., 00.10
• 10:03 The section after the Results is another protocol section ("Post-Scan Recovery: Atipamezole Injection") This post-scan recovery section should be moved to before the Representative Results section, per our video format criteria.
• 00:14 - 00:38 The segments with vertical video are not ideal for JoVE videos, we recommend you reshoot this segment in landscape mode.

Narration has been re-recorded to begin after the main title card.

The results section has been moved to after recovery.

The vertical segments have been re-shot.

10. Audio Editing and Pacing:
• Audio Levels are quite Low and not Balanced. Please ensure audio level peaks average around -9 dB.

We have re-recorded the audio and made it more balanced.

Once done please ensure that the video is no more than 15 min in length. Please upload the high resolution video
to https://www.dropbox.com/request/o6bpZAzqXYysZ0ZbnA51?oref=e

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The article describes a protocol to record long duration and stable fmri of rats under anesthesia with a mixture of isoflurane and intravenous infusion of dexmedetomidine.
A processing of data is also proposed with different free softwares commonly used by the MRI community.
The article is well written, descriptions of operations are clear and references are good.
I really appreciate the reading - and acknowledgments.
I have no comment.

We appreciate the reviewer's comments and thoughts.

Reviewer #2:
Manuscript Summary:
The article and video present and very thorough and well described protocol for carrying out resting fMRI sanning and analysis on rats. The article is very well written and the video has excellent attention to detail and is of very high quality. I have only minor comments:

Line 254 - "Previous studies have suggested 6 min is sufficient to produce functional connectivity networks" Please include references. The rs-fMRI scan time is not long enough in current standard, which would affect the reliability of the results (DOI: 10.1016/j.neuroimage.2013.05.099).

The scan duration necessary for fMRI activation detection is a function of effect size, spatial signal-to-noise ratio (SNR) and temporal SNR, as shown previously by Murphy et al. [1]. The use of a small surface coil and high magnetic field (9.4T) substantially enhances SNR and BOLD sensitivity. We previously empirically showed that a single 6-min scan can detect resting-state brain networks in rats [2]. Indeed, it has been our experience that if we do not see robust functional connectivity network in a 6-min run, it always indicates that the anesthetized rats are not under an optimal physiological conditions. However, we always scan multiple runs of resting-state fMRI scans (typically 3-4) for each rat, then average the results to derive final

statistical maps. As the reviewer pointed out, Birn et al. [3] suggested a scan time of 10 min or longer for resting-state network detection in humans. Our approach is consistent with Birn's findings in this regard.

We have revised the manuscript to further clarify this.

[1] Murphy K, Bodurka J, Bandettini PA. How long to scan? The relationship between fMRI temporal signal to noise and necessary scan duration. Neuroimage 2007;34:565–74. https://doi.org/10.1016/j.neuroimage.2006.09.032.

[2] Lu H, Zou Q, Gu H, Raichle ME, Stein EA, Yang Y. Rat brains also have a default mode network. Proc Natl Acad Sci U S A 2012;109:3979–84. https://doi.org/10.1073/pnas.1200506109.

[3] Birn RM, Molloy EK, Patriat R, Parker T, Meier TB, Kirk GR, et al. The effect of scan length on the reliability of resting-state fMRI connectivity estimates. Neuroimage 2013;83:550–8. https://doi.org/10.1016/j.neuroimage.2013.05.099.

Line 279 - Skull-stripping has been specified as the first preprocessing step but scans shown in Figure 6 have not been skull-stripped.

Figure 6 is meant to show real-time ICA analysis during the scan. This does not include skull-stripping. We have moved post-processing to the discussion section as it is beyond the scope of the protocol shown.

Line 280 - Individual rs-fMRI data were registered to a common space. Please state whether an atlas or a study-specific population template (n=?) was used as a common space.

Similarly, we have moved this section to the discussion section, but have included that we have registered our data to the space of one representative animal.

Figure 6 - Please provide the power spectrum of noise/signal components too (https://fsl.fmrib.ox.ac.uk/fslcourse/lectures/practicals/ica/index.html#cleaning).

We have provided the power spectrum in both the manuscript and video.

Section 6 - Please state versions of the software used. Also, was motion correction (e.g., MCFLIRT in FSL/MELODIC) used? If not, please justify.

We have included the versions of the software used in the materials list. We have clarified in the text that as motion correction is so minimal due to proper ear bar and bite bar placement, we have not found it to make a difference in our studies.

At the risk of imposing, additional references that might be useful to cite if the authors wish: 10.1016/j.neures.2020.05.006 because the article discusses several preprocessing steps and analysis steps that are covered only briefly in the current manuscript. The article also goes

into a bit more detail about regressing out the signal from white matter and ventricles (which is performed in the present manuscript - Line 286-288)

Thank you, we have included this reference.

In addition, because anaesthesia is a key issue that the authors emphasize, the article 10.3390/ani10061050 characterises the pharmacokinetics of medetomidine, further supporting the dose that is determined empirically in the present manuscript.

Thank you, we have also included this reference and thank the author for their thorough review and comments.

Vet Review:

#	Time in the video	comment	Change in video required Yes/No	Change in text is sufficient Yes/No	Suggested Changes
Example	2:20 – 2:34	Name of drug used for anesthesia is not mentioned	No	Yes	
1	1:15-1:28	I did not see scavenging system to protect the animal user when the induction chamber is open, and the anesthesia machine is on for the nose cone.	Yes	Yes	Scavenging system to protect the animal user needs to be mentioned or shown. Some people work under the hard-ducted hood to protect the researcher. **Response: We have clarified this in the audio script and it is noted in Figure 1 of the manuscript. Details of the**

					purchase of the benchtop scavenging system are included in the materials list.
2	1:28	What was the % of isoflurane after the animal is placed in the nose cone? The video mentioned 2.5% for the induction chamber, but not for the nose cone.	No – if the audio script can be changed without changing the video.	Yes	**Response: We have added to the audio script and text that the isoflurane concentration should remain at 2.5% when the animal is placed on the nose cone.**
3	1:30	Once the rat is anesthetized and transferred from the induction box to the nose cone before shaving the rat, eye lubricating ointment should be placed immediately to prevent dryness of cornea.	Yes	Yes/ No – depending on how well the text flows.	Suggest putting eye lubricating ointment here. **Response: We have moved putting lubricating ointment to the eye here, as suggested.**
4	3:47	It is not clear if lubricant was used for the rectal temperature probe cover. It was not mentioned or showed if the lubricant was used.	No – if the audio can be changed to describe lubricant is used for the rectal probe cover without the need	Yes	Lubricant (such as KY Jelly) for the rectal temperature probe should be used and mentioned in the video and in the text. **Response, we have clarified in the audio and the text that the probe cover is pre-lubricated.**

			to change the video.		
5	4:26-4:33	This is a comment. I have concerns that readers/ viewers may be misled. It is not required to have an aseptic field for subcutaneous injections as shown in the video. Please be advised alcohol wipe and the motion of wiping shown in the video may only remove the hair and do not create an aseptic field.	No	No	Readers/ viewers must understand that the purpose of using alcohol wipe and the motion of back and forth wiping is to remove the hair. The purpose is not to create an aseptic field. **Response: We have clarified the audio and text to this point.**
6	5:26	Eye lubricating ointment is placed here (timing is not ideal.) I would suggest the researchers confirm with their institutional attending veterinarian if the eye lubricating ointment should be placed as early as possible.	Yes	Yes	Once the rat is anesthetized and transferred from the induction box to the nose cone before hair shaving, eye lubricating ointment should be placed immediately to prevent dryness of cornea. (timing at 1:30). Ideally, the ointment should be reapply for the length of imaging (5 hours); however, I understand that for this imaging procedure, reapplication of lubricating eye

					ointment is not possible. **Response: We have re-shot the video to put lubricating ointment on the eyes at the beginning, as suggested.**
7	10:19	Please confirm the gauge size of the intramuscular injection needle. It appeared too large for the rat in the video.	Yes - if the needle size needs to be changed.	No	Recommended needle size should be 25-27 gauge. **Response. The video shows a 25 gauge needle. The audio and the text have been updated to note this.**

For the text,

Line 119 - Please specify if the PE50 line used for subcutaneous infusion was sterile? Please specify if the subcutaneous fluid solution was sterile. They should be sterile.

We have specified in the text that the PE50 line and subcutaneous fluid solution are sterile.

Line 123-125 – It is good that the dose (mg/kg) is described in the text. Please specify the strength of dexmedetomidine after dilution from the stock solution. The final concentration of the diluted dexmedetomidine should be described in the text. It is 0.025 mg/ml.

We have specified the concentration of the diluted solution.

Line 127-128 – Please specify the strength of atipamezole after dilution from the stock solution. The final concentration of the diluted atipamezole should be described in the text. It is 0.05 mg/ml.

We have specified the concentration of the diluted solution.

Line 129 – please specify if the glass bottle used was sterile. It should be sterile.

We have specified that a sterile glass bottle was used.

Line 149-152 – there is not description or set up of anesthetic scavenging system to protect the researchers/ animal users. It appeared that the animal user was exposed to the inhalant isoflurane when the induction chamber was open and from the nose cone when the anesthetic machine was turned on.

We have described the use of the scavenging system and included it on our materials list. The benchtop system actively scavenges waste gases from each of the areas where isoflurane is delivered.

Line 156-157 – please specify the gauge of the needle used for IP injection and describe the location of the IP injection (lower right quadrant of the abdomen as shown in the video).

We have specified the gauge of the needle used and described the location of the injection.

Line 180 -

Rectal probe cover (the same as the video) – please specify if lubricant such as KY jelly was used on the probe cover.

We have clarified in the audio and the text that the probe covers come pre-lubricated.

Line 199

It is not required to have an aseptic field for subcutaneous injections. Please be advised alcohol wipe and the motion of wiping shown (back and forth) in the video may only remove the hair and do NOT create an aseptic field.

We have clarified in the audio and text the purpose of the alcohol swab.

Line 208

Ideally, eye lubricant should have been placed immediately after the animal was placed on the nose cone before hair shaving. It was not clear why the researcher/ animal user waited until this step to lubricate the eyes. Please specify what was used to lubricate the eyes. Was it artificial tear ointment?

We have re-recorded video to show the eye lubrication placed immediately on the animal's eyes. We have specified the ointment and included it in the materials list.

Line 272 - 273

It is good that the dose (mg/kg) is described in the text. In addition, please specify the volume of Atipamezole used in parenthesis with the concentration (0.05 mg/ml). Please specify the size of the needle gauge for the IM injection.

We have specified the volume and concentration of atipamezole, as well as the gauge of needle for the IM injection.