

TRIDENT Marmoset Core Standard Operating Procedures

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Marmoset Standard Operating Procedures

Title: Surgery Protocol for Pre-Formed Fibril (PFF) injection

SOP No.: TRIDENT_RP_marmPFFinjection-V1

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Created: For TRIDENT, by Dr. Alessandro Zanini (Western site, Everling Lab), Jiayue Yang and Dr. Margaux Teil (McGill site, Clery Lab). Protocol comparison by Sriram Jayabal, Research Program Director, TRIDENT.

Surgery Protocol for Pre-Formed Fibril (PFF) injection

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Pre-formed Fibrils (PFF) Injection particulars

Location of injection: (From Paxinos Atlas)

The coordinates used for injection were taken directly from the Paxinos atlas. Similar coordinates were previously used by the Japanese group (Shimozawa et al., 2017):

- CAUDATE:
 - A 11.0 mm
 - L 3.0 mm
 - Z 6.0 mm
- PUTAMEN:
 - A 11 mm
 - L 5.5 mm
 - Z 6.5 mm
- Marmoset PFFs used were procured from Dr. Thomas Durcan, TRIDENT iPSCs and Organoids Core lead at EDDU, McGill at a concentration of 5 mg/ml concentration in 80 uL aliquots (80 uL/marmoset is calculated such that 50 uL is for injection and the remaining 30 uL is excess in case of wastage or spill).
- PFF amount to be injected unilaterally (left side): 25 uL per site (that is 25 uL at Putamen and 25 uL at Caudate; 50 uL total/marmoset) at a flow rate of 1 uL/min.

Notes: PFFs requests are usually made at least 6 weeks prior to the injection date to allow for the EDDU team at McGill to get them ready and to have them transported to the sites. The shelf-life recommendation from Dr. Durcan is to use it within 1- 2 months, ideally. Keep them stored at -80 °C.

Some pilot animals used a different set of coordinates, i.e., for caudate -A 11.5 mm; -L 3.0 mm & -Z 6 mm and for the putamen -A 11.5 mm; -L 5.5 mm & -Z 7.5 mm

Western Site

Materials and equipment

- Stereotactic frame (Narishige, Model SR-6C-HT)
- Disposable microbrush (Bisco Schaumburg, Illinois, USA, Item number: X-80250P)
- Ultraviolet dental curing light (Dental Power, Dental Wireless Cordless LED curing light, reference: lux60)
- Resin composite (Bisco, Schaumburg, Illinois, USA, Core-flo DC Lite, item number: A-17801P)
- Syringe pump (SAI Infusion Technologies, Baxter AS50 Pump – refurbished)
- Pump for water blanket (Palm Industrial, Canada, Gaymar T Pump 07999-000)
- Pump for forced-air blanket (Southwest Medical, Oklahoma, USA, 3M Bair Hugger Model 505 Patient Warming System)
- Metal brush (Gesswein Canada, Supra ME wire end brushes, straight wire, item number 110-8791)
- Wound clips (9 mm, Fine Science Tools, Item number 12022-09)
- Anesthesia mask (custom-made, CAD file: <https://zenodo.org/records/8045220>)
- Tear Gel
- Motorized drill with sterile drill bits
- Hamilton 50ul gastight syringe (Hamilton, PN: 80920), 30G needle
- Integrated Stereotaxic Injector (53315 Integrated Stereotaxic Injector - Single, Stoelting) connected to PC or tablet running ISI software
- Bone wax

Procedures

1. Administer maropitant citrate (Cerenia, 1 mg/kg, 10 mg/mL) subcutaneously to the marmoset 20 minutes prior to sedation, to reduce nauseousness, salivation, and bronchial secretions.
2. Administer Tylenol (10 mg/kg, 80 mg/mL) orally
3. Induce anesthesia with intramuscular injection of ketamine (15-20 mg/kg, 100 mg/mL) plus medetomidine (0.025 mg/kg, 1 mg/mL) mixed in the same syringe.
4. Place a catheter (gauge 24) in one of the lateral tail veins.



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5. Inject a first load of propofol through the catheter (2-5 mg/kg, 10 mg/mL)
6. Via a syringe pump, perform a continuous rate infusion of propofol (0.3-0.9 mg/kg) mixed 50-50 in 0.9% saline solution (diluted to 5 mg/mL: 5 mL propofol, 5mL saline). Flow rate is: (weight in kg * dose)/concentration. For example: $(0.4 \text{ kg} * 0.3 \text{ mg/kg}) / 5 \text{ mg/mL} = 0.024 \text{ mL/min}$
7. To maintain general anesthesia during the surgical procedure, deliver gaseous isoflurane (0.5-3.0%) in oxygen (1.5-2 L/min) via a custom-designed 3D printed mask (see Key Resources table in Zanini et al., 2024, STAR Protocols, for CAD file), attached to the stereotactic frame.
8. To combat inflammation, give a loading dose of metacam (0.2 mg/kg, 5 mg/mL) subcutaneously or orally (1.5mg/mL).
9. Deliver intravenously an antibiotic Cefazolin (22 mg/kg, 334 mg/mL) diluted by 10 in 0.9% saline solution at the beginning of the surgical procedure and every 90 minutes.
10. Place a small amount of sugar paste under the animal's tongue to prevent hypoglycemia during the surgery.
11. Protect the eyes using Tear-Gel (or equivalent).
12. Maintain body temperature using a circulating water blanket, a forced-air warming blanket, and fleece blankets placed under the sterile surgical drape.
13. Monitor the temperature, heart rate, respiration rate, blood pressure, and SpO₂ throughout surgical procedure.
14. Shave the head of the animal to keep the surgery area clearly visible.
15. Place the animal in the stereotactic frame (Narishige, Model SR-6C-HT).
16. Clean the scalp with alcohol, to remove excessive dirt and create aseptic conditions around the incision site.
17. Perform a midline incision along the cranium and retract the temporalis muscle to gain access to the underlying periosteum.
18. Carefully remove the periosteum and any other residual tissue from the skull. To accomplish this, clean the skull surface using hydrogen peroxide (2%) and cotton swabs.

Note: Since the skull of the common marmoset is small and extremely fragile, the use of fixation screws is not recommended. Furthermore, the small size of these animals is well suited to the



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use of fixation methods based only on adhesive strength. For this reason, we have employed a surgical procedure which involves the use of cement typically used in dental restoration. However, to ensure the effectiveness of this type of implant, it is critical to carefully prepare the surface of the skull to achieve optimal bonding of the dental cement.

19. Stop any residual bleeding around the skull using 15.5% ferric sulfate hemostatic gel (or equivalent).
20. Using a small metal brush, gently abrade the skull surface.
21. Rinse the skull surface thoroughly with sterile saline, then dry thoroughly all fluids and remove all bone residue using absorbent cotton swabs.
22. Using a microbrush (Bisco disposable regular applicator, item number: X-80250P; Bisco Schaumburg, Illinois, USA), apply two layers of adhesive resin (All-bond Universal, Bisco, Schaumburg, Illinois, USA) on the skull.
23. Air dry the resin (you can use a sterile empty 30cc syringe).
24. Cure the surface with an ultraviolet dental curing light (Dental Power).
25. Repeat steps 20 and 22 (no need to air dry).
26. Using a pointer mounted on the stereotactic arm, locate the two sites of injections (Anterior = 11.0. For left caudate: Lateral = 3.0 and Depth = -6 from the brain surface. For left putamen: Lateral = 5.5 and Depth = -6.5 from the brain surface).
27. Drill the first hole in the skull until reaching the brain surface (tested with a dental probe or equivalent).
28. Cover the first hole with wet gauze, then repeat the drilling for the second injection site.

Note: *A difference in procedures emerges at this point depending on the type of injection. If the animal is injected with saline (control animal), step 28 is directly followed by step 29. If the animal is injected with pre-formed fibrils of alphasynuclein (PFF animal), the injection must be performed under a ventilated cabinet. Thus, in between step 28 and 29, the entire stereotactic frame and the animal are moved under the cabinet.*

29. Mount the filled syringe (Hamilton 50ul gastight, 30G needle) on the Integrated Stereotaxic Injector (53315 Integrated Stereotaxic Injector - Single, Stoelting), directly connected to a Windows tablet running ISI software.
30. Lower the needle tip until it reaches the brain surface in the first drill site, then lower it further



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(6 or 6.5mm depending on the injection site) to reach the injection site.

31. Wait 3 minutes after the tip of the needle reaches the injection site.
32. Launch the first injection (25ul, rate of 1ul per minute).
33. At the end, wait 5 minutes before retracting the needle.
34. Clean gently the tip of the needle with alcohol, then repeat steps 30 to 33 for the second injection site.

Note: *If the animal is injected with PFF, step 35 is performed in the cabinet, and the animal is moved back on the surgery table only after step 35.*

35. Seal the two holes in the skull with bone wax.
36. Place the head post on the stereotactic manipulator, ensuring its correct orientation and position on the marmoset's head.

Note: The head post is designed based on a computerized tomography (CT) scan of a marmoset head. To determine the correct position of the head post, locate the vertex of the marmoset's skull, which is situated halfway between the nasion and inion (rostral-caudal direction) and between the right and left pre-auricular points. The center of the head post, where the fastening screw is present, should coincide with this vertex.

37. Lower the head post onto the skull using the stereotactic manipulator.
38. Apply the resin composite (Core-flo DC Lite, Bisco, Schaumburg, Illinois, USA) on the layer of adhesive resin and on the bottom of the head post.

Note: *Our head post is machined from solid polyetheretherketone, or PEEK. The related CAD file has been made publicly available (see Key Resource Table in Zanini et al., 2024, STAR Protocols, for source). In the base of our head post we designed several small holes, to allow the cement to fill in during the surgery and ensure better adhesion to the skull. When the marmosets are not imaged or in training, a PEEK screw is inserted in the head post, to keep debris from entering the screw threads.*

39. Using a fine tip applicator, fill the holes in the base of the head post with the resin composite to ensure a better adhesion to the skull.



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40. Smoothen the adhesive cement around the external edges of the head post, as sharp edges may irritate or lacerate the surrounding tissues.
41. Cure the adhesive with ultraviolet dental curing light (Dental Power).
42. Suture the margins on the front and/or on the back of the head post, to ensure a tight contact between the cement and the edges of the wound.
43. Post-operatively, administer buprenorphine (0.005-0.01 mg/kg, 0.3 mg/mL) intramuscularly once the animal starts to wake up, then repeat after 6-8 hours post-surgery.
44. Administer Metacam (0.1 mg/kg) orally once a day for 5 days for pain management plus infants Acetaminophen (Tylenol) (10 mg/kg, 80 mg/mL) orally twice daily for 5 days.

Note: *To ensure good adherence of the head post and to allow the animal to recover following the surgical operation, it is advisable to wait at least 2 to 3 weeks before starting the MRI training inside the body restraint. During this period, check the animal health and in particular the skin edges approximating the dental cement must be reviewed on a daily basis at minimum.*

Contributed by Dr. Alessandro Zanini (Everling Lab).

McGill Site

Materials and equipment

- Brainsight robot combined with computer and stereotaxic frame (Rogue Research)
- 100 µl Hamilton syringes with 22G needle; Hamilton Co., Ref # 1710 RN
- 26sG Hamilton needles; Hamilton Co., Ref #775802
- Motorized pump and injector
- Motorized drill with sterile drill bits

Procedures

1. The marmoset arrives in the surgical room in a transfer box. They are restrained and injected intramuscularly (IM) with alfaxalone (8mg/kg). The marmosets are put back in their transfer boxes for around 10-15 minutes.
2. Once asleep, the animal is removed from the transfer box and injected subcutaneously (SC) with Cerenia (1mg/kg) and Slow-Release Buprenorphine (0.2mg/kg).
3. To keep the animal hydrated, a catheter can be placed in the tail vein with an NaCl 0.9% injection rate of 10mg/kg/hr. If not, the animal is SC injected with 5ml NaCl 0.9% before the surgery.
4. The marmoset's mouth is opened, and lidocaine is sprayed in the back of the throat to locally anaesthetise before intubation.
5. The animal is intubated with a 2mm intubation tube. Correct intubation is verified before the animal is plugged to the anaesthetic machine. Isoflurane is administered at a flow rate of around 1.5-2% on 1L/min O₂.
6. The eyes of the marmoset are protected using Teargel.
7. The head of the marmoset is shaved (tufts included) and a local anaesthetic is injected cranially beneath the skin of the skull (Lidocaine = 0.05ml, 20mg/ml).
8. Set up the monitoring system for the animal to monitor vitals closely during the whole surgery (temperature, SPO₂, heart rate).



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9. The marmoset is placed on the stereotaxic frame with ear bars, pallet holder, and eye bars. Making sure that the intubation tube is not stuck between the pallet holder and the pallet of the marmoset. The head of the animal is placed symmetrically in the middle of the frame (verified with measurements on ear bars).
10. The area of incision on the head of the marmoset is cleaned with chlorhexidine and alcohol to insure an aseptic incision site.
11. To keep the marmoset's body heat stable during the surgery, the animal is covered with a heating blanket and placed on a heat pad.
12. Cover the animal with a sterile drape, only leaving the aseptic incision site uncovered.
13. Make an incision at the vertex of the skull using a scalpel blade. The skull will be scraped of tissue and muscles to clean the skull for the Brainsight laser grid. Sterile saline can be used to remove blood and debris.
14. Next, the Brainsight robot will execute the laser grid and 3D overlay before the surgery can continue. Once the Brainsight steps are completed, drilling and injection steps may proceed.
15. Place the drill on the arm of the robot. Progressively lower drill and synchronize drilling with veterinary staff to only drill through the skull. Remove drill once hole is made. Verify that the skull is completely drilled through using a 25/26G needle.
16. Set up the sterile Hamilton with the 26G needle and connect to the injector. Fill the needle with the required preformed fibrils. Make sure there are no bubbles in the syringe and lower level to remove one drop of the liquid to assure the lack of bubbles in the needle.
17. Once ready for injection, place the injector on the arm of the robot. Lower progressively until reaching the brain while checking that the needle and hole are well aligned.
18. Once in the brain, lower slowly until reaching the first site of injection. Start injection of 25 μ l at the rate of 1 μ l/min. Once injection is complete, keep the needle in place for 5 minutes and slowly retract to next injection site.
19. NB: During the injections, hydrate the skull with sterile saline and cover with sterile gauze.
20. Repeat the previous steps until the end of the final injection. Once all injections are completed and the wait times are done, slowly retract the needle out of the marmoset's brain.
21. Suture the incision using Prolene sutures (4-0). Once sutures are completed, the

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closed incision site is glued with surgical glue to avoid wound opening.

22. Remove the marmoset from the stereotaxic frame and turn off the isoflurane, leaving oxygen on until the marmoset breathes on his own. When the animal starts to wake up and coughs, the intubation tube can be removed, while leaving the animal on a heating pad or the incubator during recovery.
23. Once the marmoset is fully awake, it can be placed back in its transport box and returned to its home cage.
24. During the next days, the animal is closely monitored and given an enriched diet (ex: mazuri slurry and ensure) once a day. Weight is monitored closely two and four days after the surgery, as well as the animal's behavior (mobility, eyes, grooming, etc). Tylenol is given twice a day to the animal to reduce inflammation.
25. If the animal has an inflammation (red and swollen of the suture), the animal is taken out and receive a Ceftiofur (Excede) antibiotics. If no inflammation observed and the animal is recovering well, no antibiotics is needed for post-surgery care.

The protocol was adapted from the Huot lab. Contributed by Dr. Margaux Teil (Huot Lab) and Jiayue Yang (Clery lab)



COMPARISON OF THE MARMOSET SURGERY PROTOCOL BETWEEN MCGILL AND WESTERN SITES

Note: This section aids in direct comparison of the two protocols broadly and is for informational purposes only. For precise instructions, refer to the individual core protocol when doing the procedure.

PRE-SURGICAL PREPARATION

1. Initial anesthesia Induction:

○ Western Site:

- Administer Cerenia (maropitant citrate, 1 mg/kg Subcutaneous (SC)) 20 minutes before sedation to reduce nausea, salivation, and bronchial secretions.
- Administer Tylenol (acetaminophen, 10 mg/kg, 80 mg/mL) orally
- To induce anaesthesia, administer ketamine (15-20 mg/kg IM) plus medetomidine (0.025 mg/kg IM) mixed in the same syringe.

○ McGill Site:

- The marmoset arrives in the surgical room in a transfer box. They are restrained and injected intramuscularly (IM) with alfaxolone (8mg/kg). The marmosets are put back in their transfer boxes for around 10 - 15 minutes.
- Once asleep, the animal is removed from the transfer box and injected subcutaneously (SC) with Cerenia (1mg/kg) and Slow-Release Buprenorphine (0.2mg/kg).

2. Fluid Management:

○ Western Site:

- Place a 24G catheter in a lateral tail vein and maintain fluids using continuous rate infusion of propofol (0.3-0.5 mg/kg) mixed 50-50 in 0.9% sterile saline solution (diluted to 5 mg/mL: 5 mL propofol, 5mL



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saline). Flow rate is: (weight in kg * dose)/concentration. For example:

$$(0.4 \text{ kg} * 0.3 \text{ mg/kg}) / 5 \text{ mg/mL} = 0.024 \text{ mL/min}$$

- **McGill Site:**

- Place a tail vein catheter for sterile NaCl (0.9%) infusion at 10 mg/kg/h, or administer 5 mL SC NaCl (0.9%) if no catheter is placed. If a tail vein catheter is not used, the animal is SC injected with 5ml sterile NaCl 0.9% before the surgery.

3. General anaesthesia:

- **Western Site:**

- To maintain general anesthesia during the surgical procedure, deliver gaseous isoflurane (0.5-3.0%) in oxygen (1.5-2 L/min) via a custom-designed 3D printed mask (see Key Resources table in Zanini et al., 2024, STAR Protocols, for CAD file), attached to the stereotactic frame.

- **McGill Site:**

- The marmoset's mouth is opened, and lidocaine is sprayed in the back of the throat to locally anaesthetize before intubation.
- The animal is intubated with a 2mm intubation tube. Correct intubation is verified before the animal is plugged to the anaesthetic machine. Isoflurane is administered at a flow rate of around 1.5-2% on 1L/min O2.

4. Pre-Surgical Analgesia & Antibiotics:

- **Western Site:**

- To combat inflammation, give a loading dose of the metacam (0.2 mg/kg, 5 mg/mL) subcutaneously.
- Administer IV cefazolin (22 mg/kg, 334 mg/mL, diluted 1:10 in 0.9% sterile saline) at the beginning of the surgery. Readminister cefazolin every 90 minutes.

- **McGill Site:**



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- Analgesic already administered during initial anesthesia. If the animal has an inflammation (red and swollen of the suture), the animal is taken out and receive a Ceftiofur (Excede) antibiotics. If no inflammation observed and the animal is recovering well, no antibiotics is needed for post-surgery care.

5. Protective Measures & Monitoring:

- **Western Site:**
 - Place a small amount of sugar paste under the animal's tongue to prevent hypoglycemia during the surgery.
 - Protect the eyes using Tear-Gel (or equivalent).
 - Maintain body temperature using a circulating water blanket, a forced-air warming blanket, and fleece blankets placed under the sterile surgical drape.
 - Monitor the temperature, heart rate, respiration rate, blood pressure, and SpO₂ throughout surgical procedure.
- **McGill Site:**
 - Apply Tear-Gel to the eyes.
 - Maintain body temperature using a heating pad. Warming blankets are also placed on the stereotaxic.
 - Monitor temperature, SpO₂, and heart rate.

SURGICAL PROCEDURE

6. Positioning and Aseptic Preparation:

- **Western Site:** Place the animal in a stereotactic frame (Narishige SR-6C-HT).
- **McGill Site:** Use a Brainsight robot combined with a stereotactic frame (Rogue Research).
- **Both Sites:**
 - Shave the head.
 - Clean the incision site with alcohol (Western) or chlorhexidine + alcohol (McGill).



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- Cover the body with a sterile drape, exposing only the surgical site.

7. Incision and Skull Preparation:

- **Both Sites:**
 - Make a midline incision.
 - Clean the skull using sterile saline and swabs.
- **Western Site:**
 - Use hydrogen peroxide (2%) to clean residual tissue.
 - Stop bleeding with 15.5% ferric sulfate hemostatic gel.
 - Abrade the skull with a small metal brush (needed to house the headpost).

8. Drilling and Injection Preparation:

- **Western Site:**
 - Drill two holes at the predefined stereotaxic coordinates.
 - If injecting PFFs, move the animal to a ventilated cabinet before injection.
 - Hydrate the skull with sterile saline and cover it with sterile gauze during the procedure.
- **McGill Site:**
 - Use a motorized drill with sterile drill bits.
 - Verify the hole using a 25/26G needle.
 - Hydrate the skull with sterile saline and cover it with sterile gauze during the procedure.

9. Injection Procedure:

- **Western Site:**
 - Use a Hamilton 50 μ L gastight syringe (30G needle) with an integrated stereotaxic injector.
 - Inject 25 μ L at 1 μ L/min, waiting 3 minutes before injection and 5 minutes before retracting.
- **McGill Site:**
 - Use a 100 μ L Hamilton syringe (22G needle).



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- Inject 25 μ L per site at 1 μ L/min, waiting 5 minutes before retracting.

POST-SURGICAL CARE

10. Wound Closure & Headpost Placement (Western Only):

- **Western Site:**
 - Seal holes with bone wax.
 - Position a PEEK headpost using resin composite (Core-flo DC Lite, Bisco).
 - Cure with ultraviolet dental curing light.
 - Suture margins around the headpost and apply surgical glue.
- **McGill Site:**
 - Suture using Prolene 4-0 and apply surgical glue.

11. Pain Management & Recovery:

- **Western Site:**
 - Administer buprenorphine (0.005-0.01 mg/kg, 0.3 mg/mL, IM) upon wake-up, repeated after 6-8 hours.
 - Administer metacam (0.1 mg/kg PO daily for 5 days) and acetaminophen (Tylenol, 10-20 mg/kg, 80 mg/mL PO, BID for 5 days).
- **McGill Site:**
 - Monitor recovery on a heating pad until fully awake, then return to the home cage.
 - If the animal is found having inflammation (red and swollen) around the suture, the animal will receive an antibiotics Ceftiofur (Excede). Otherwise, administer acetaminophen (Tylenol, 0.8 – 1 mL, 160mg acetaminophen per 5ml) twice a day.

SUMMARY OF KEY DIFFERENCES:

The Western and McGill sites take slightly different approaches to anesthesia, surgical preparation, and recovery care.

Anesthesia

Both rely on isoflurane to maintain anesthesia during surgery, but their induction methods differ. The Western site begins with an intramuscular injection of ketamine and medetomidine, transitioning to isoflurane via a custom 3D-printed mask. The McGill site, on the other hand, uses glycopyrrolate and alfaxalone, followed by intubation with isoflurane after applying lidocaine to the throat.

Surgery

When it comes to preparing for surgery, the Western site prefers a Narishige SR-6C-HT stereotactic frame, whereas McGill employs a Brainsight robotic system for added precision. Skull preparation is another key difference. The Western site uses hydrogen peroxide and ferric sulfate to clean the skull and control bleeding before lightly abrading the bone surface with a small metal brush. In contrast, McGill follows a laser grid system to map injection sites and clean the skull accordingly.

One major distinction is the implantation process. The Western site places a PEEK headpost, securing it with resin composite and UV curing, while McGill does not use a headpost and instead closes the surgical site with sutures and surgical glue.

Recovery

Post-surgery, pain management also varies slightly— Western administers buprenorphine, whereas McGill relies on meloxicam for inflammation and pain relief. The general procedure is overall very similar but with customized additions at both the cores, while the overall shared goal is to ensure the best surgical outcome for marmosets.

Comparison of the protocols was done by Sriram Jayabal, the Research Program Director-TRIDENT.

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Shimozawa, A., Ono, M., Takahara, D. et al. Propagation of pathological α -synuclein in marmoset brain. acta neuropathol commun 5, 12 (2017).
<https://doi.org/10.1186/s40478-017-0413-0>



Marmoset Core SOPs

Marmoset Standard Operating Procedures

Title: Pairwise Visual Discrimination (PVD) Task

SOP No.: TRIDENT_RP_marmPVD-V1

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Created: For TRIDENT, by Dr. Alessandro Zanini (Western site, Everling Lab) and compiled by Dr. Sriram Jayabal (Research Program Director, TRIDENT).



Pairwise Visual Discrimination (PVD) Task

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Introduction

The PVD task consists of two phases: associative learning and reversal learning of the reward contingencies, thereby evaluating cognitive flexibility in response to changing rules.

PVD-Learning Phase: The marmosets choose between the reward (S+) stimulus and the unrewarded (or distractor, S-) stimulus, which are randomly displayed on the left or right side of the touchscreen. A correct response is rewarded by water-diluted maple syrup or marshmallow fluff (or another preferred reward specific to the marmoset in training).

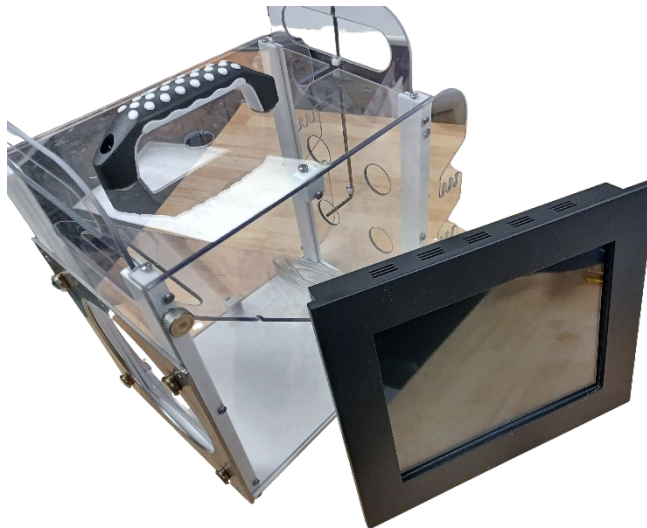
PVD-Reversal Phase: When marmosets fully learn the task and achieve at least 90% accuracy (i.e. > 27/30 trials correct within max 1 hour; in some cases, depending on the performance of a specific animal, the threshold for reversal is set at 80%, i.e., >24/30 trials) for 2 consecutive days, the reversal phase starts by reversing the conditional rule to assign the reward to S- stimuli.

This protocol describes the procedures followed at Western University Marmoset Core of TRIDENT.

MATERIALS

Apparatus:

In-cage touchscreen testing using Raspberry Pi-based Marmtouch software



Western Touch Screen Box

Software:

- i. MATLAB and MonkeyLogic (customized version 2.2.32)
- ii. Python and R (for analyses)

PROCEDURES**1. GENERAL HARDWARE CHECK**

- i. The hardware is quickly tested prior to every training or testing session to ensure the program is running properly. This must be done before the marmoset is in the box/chair to prevent returning the marmoset without training due to technical difficulties.
- ii. The items to check include:
 - 1) the Touchscreen box is attached to the home cage,
 - 2) the reward pump delivers the reward,
 - 3) the touchscreen is sensitive and responding to the touches, and
 - 4) the task shows expected outputs (visual stimuli are correctly displayed, and reward is correctly delivered) and inputs (touching the correct stimulus triggers the reward, touching the incorrect stimulus triggers the punishment – longer ITI).
- iii. Training/testing schedule:

Marmosets perform one training session per day at the Western site, usually in the morning, before the feeding routine.

2. PRE-TRAINING PROCEDURES**Phase 1: Acclimation*****a. Aim:***

Acclimation allows marmosets to habituate to the new apparatus and to enter the touchscreen box. And further allows marmosets to associate touching the screen with acquiring a reward from the delivery pump.

Note: Exhibit caution and do not allow marmosets to sit idle and do nothing in the box. The marmosets need to associate the box to doing the task (work) to get their reward.

b. Criteria:

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- i. Marmosets spend approximately 45 minutes in the touchscreen box. Marmosets seem calm and motivated to remain in the box. A criterion for progressing to the next phase is that the marmoset remains in the box to consume the reward, rather than running back and forth to the home cage, as such behaviour may indicate fear or discomfort with the testing environment.
- ii. Marmosets complete 30 trials within 45 minutes.

c. Instructions:

- i. The Touchscreen box is attached to the marmoset home cage.
- ii. Exploratory behavior is encouraged by sticking marshmallow pieces on the screen and enrichment objects in the box.
- iii. Display a visual stimulus on a black background centered on the screen. Visual stimulus must be something attractive for the marmoset (marmoset face, pictures of marmosets grooming/fighting, food).
- iv. The marshmallows on the screen attract the monkey. Reward marmosets for touching the presented stimuli.
- v. Inter-trial interval (ITI) set at 5 seconds.
- vi. To avoid distressing the marmosets, the door to the Touchscreen box is not closed. This allows the marmosets to freely move in and out of the Touchscreen Box.

Phase 2: Unguided Touch-Reward Association***a. Aim:***

For marmosets to associate touching the screen with acquiring a reward from the delivery pump.

b. Criteria:

Marmosets complete 30 trials of successful touch and reward acquisition within 45 minutes.

c. Instructions:

- i. The Touchscreen box is attached to the marmoset home cage.
- ii. The same task and stimuli of the previous phase are presented to the marmoset.
- iii. Enrichment objects and marshmallows on the screen are gradually removed from the Touchscreen Box, until there are no objects or marshmallows left.

The aim is to have them touch the screen autonomously, without a marshmallow as a guide.

Phase 3: Locational Touchscreen Learning

a. Aim:

Marmosets should learn to associate the visual stimuli with a reward by touching different and specific parts of the touchscreen where the visual stimuli are displayed.

b. Criteria:

Marmosets complete 30 trials of successful touch and reward acquisition within 45 minutes.

c. Instructions:

- i. The Touchscreen box is attached to the marmoset home cage.
- ii. A new task is presented, displaying two squared stimuli (100x100 pixels) on the right and left sides of the screen. Stimuli do not need to be attractive per se: the marmosets now know by touching the screen they can obtain the reward.

d. Workflow of the task:

- i. Reward marmosets for touching one of the presented stimuli (both stimuli represent a correct response). 30% chance of a bonus (double reward).
- ii. Inter-trial interval (ITI) at 5 seconds.
- iii. Time-out of 2 minutes: if the marmoset does not touch the stimuli before the time-out, the ITI is triggered and a next trial begins.

Phase 4: Pairwise Visual Discrimination Learning (Discrimination Learning)

a. Aim:

For marmosets to learn which of the two stimuli is rewarding by improving their accuracy of associating rewarded stimuli with more training.

b. Criteria:

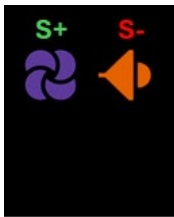
90% accuracy (namely 27 correct responses out of 30 trials presented; or any other desired level of accuracy but no lower than 80%; a response is considered correct only if the marmoset touches the S+ stimulus) over 2 consecutive days of training, with 60 minutes to 90 minutes for 3 marmosets (30 to 60 minutes per marmoset, depending on the animal needs) training time per day.

c. Instructions:

- i. The Touchscreen box is attached to the marmoset home cage.
- ii. Launch the PVD task
 - Tap on the Marmtouch icon
 - On the right-side menu, tap on Tasks
 - Select the task (Reversal)
 - Select the monkey (stimuli pairs can be monkey-specific)

d. Workflow of the task:

- i. The animal identity that is performing the task is pre-determined before they enter the box.
- ii. After scanning, two high-contrast PVD stimuli will be presented on the touchscreen simultaneously on either side of the screen (100x100 pixels). One of the stimuli will be rewarding (S+), and one will not be (S-). When touching the S+ stimuli, the marmosets will be given 0.075 mL of the reward (increasable if specific marmosets need it).



- iii. After the selection of stimuli, the correct response will persist on the screen for 1 second, while the incorrect response disappears. This fosters the animal's attention on the correct stimulus.
- iv. The default ITI is 4 seconds for correct responses. For the incorrect responses, ITI can be set longer (~5-6 seconds) to motivate the marmosets to learn the right choice. If the marmosets are making choices randomly without associating the stimuli with the reward, only in those marmosets, the ITI is set longer to a maximum of 9 seconds (longer ITI leads to longer wait time for reward and hence will motivate the marmosets to learn).

Phase 5: Pairwise Visual Discrimination Reversal (Reversal Learning)**a. Aim:**

For marmosets to learn which of the two stimuli is rewarding after a reversal. This task aims to investigate the cognitive flexibility of the common marmoset.

b. Criteria:

90% accuracy over 2 consecutive days of training, (30 to 60 minutes per marmoset) training time per day.

c. Instructions:

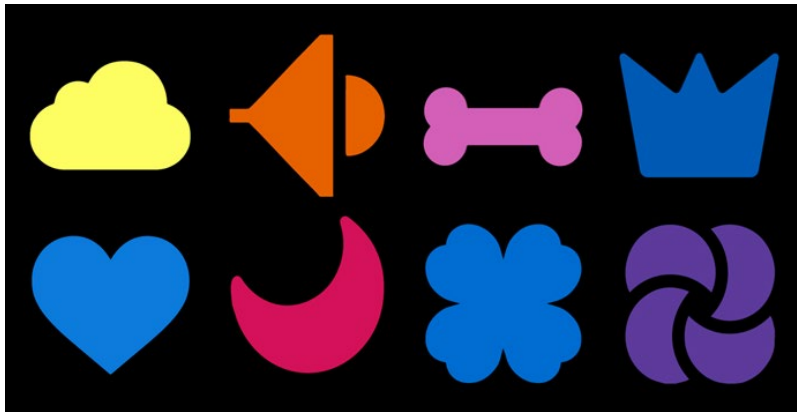
- i. The Touchscreen box is attached to the marmoset home cage.
- ii. The PVD task is launched, after reversing the association between visual stimuli and reward.

d. Workflow of the task:

- ii. The task procedures are identical to **the previous PVD learning phase**. However, the contingency of reward association of the 2 PVD stimuli will be reversed: the stimulus previously rewarded (S+) will not be rewarded anymore, while the previously unrewarded stimulus (S-) will now be rewarded.

3.DATA COLLECTION PROCEDURES***Phase 6: Baseline and beyond***

- a. After completing phases 4 and 5, the marmosets must have learned the PVD task with both the learning and reversal phases. Thus, they are considered ready to proceed with the data collection.
- b. Each data collection PVD session consists of a Discrimination Learning and Reversal Learning phase, using the same pair of stimuli, with the stimulus-reward association reversed during the reversal phase.
- c. Between each PVD cycle, the pair of stimuli will be changed to prevent repetitive stimuli that may confuse the marmosets. The stimuli are shown in the figure below.
- d. The criteria, instruction, and workflow of the task during data collection will be identical to **phases 4 and 5 of the PVD training**.



General Notes:

- The strategy of putting the reward on the touchscreen aims to keep the motivation of the marmosets, which will not be used to guide their choices during the touchscreen PVD task. It is cage/marmoset dependent and not applied on every cage/marmoset that performs the PVD task.
- Do not allow marmosets to be in the touchscreen box if the touchscreen is not responsive or if the reward is not dispensing properly.
- If marmosets seem resistant to the initial training phases, you may initially reduce the ITI to encourage marmosets to participate in the tasks. E.g., an ITI of 2 seconds instead of 5 seconds, and then slowly increase the ITI until it reaches the prescribed criteria. For some animals, 4 seconds ITI can be used as final ITI, in order to foster their task engagement.
 - Steps: 2s, 3s, 4s, 5s
- If marmosets seem to struggle moving to the next phase of the training, additional, “halfway” tasks may be employed to bridge between phases. Moreover, colorful stimuli can be used to attract marmosets during the tasks. However, all adaptations and changes of the PVD training and task need to be added to this current protocol.
- Maple syrup juice reward:
 - 35g of maple syrup.
 - 1:7 ratio of maple syrup to water (by volume).
- Marshmallow fluff reward:
 - 15g of marshmallow fluff.
 - 60g of water to mix well with the fluff.

Contributed by Dr. Alessandro Zanini (Everling Lab) and compiled by Dr. Sriram Jayabal (Research Program Director-TRIDENT).



Marmoset Core SOPs

Marmoset Standard Operating Procedures

Title: Pairwise Visual Discrimination (PVD) Task

SOP No.: TRIDENT_RP_marmPVDmultisite-V1

Date: 30 September 2025

Date last updated: 30 September 2025

Created: For TRIDENT, by Dr. Alessandro Zanini (Western site, Everling Lab) and Jiayue Yang (McGill site, Clery Lab), and compiled by Dr. Sriram Jayabal (Research Program Director, TRIDENT).



Pairwise Visual Discrimination (PVD) Task

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Introduction

The PVD task consists of two phases: associative learning and reversal learning of the reward contingencies, thereby evaluating cognitive flexibility in response to changing rules.

PVD-Learning Phase: The marmosets choose between the reward (S+) stimulus and the unrewarded (or distractor, S-) stimulus, which are randomly displayed on the left or right side of the touchscreen. A correct response is rewarded by water-diluted maple syrup or marshmallow fluff (or another preferred reward specific to the marmoset in training).

PVD-Reversal Phase: When marmosets fully learn the task and achieve at least 90% accuracy (i.e. > 27/30 trials correct within max 1 hour; in some cases, depending on the performance of a specific animal, the threshold for reversal is set at 80%, i.e., >24/30 trials) for 2 consecutive days, the reversal phase starts by reversing the conditional rule to assign the reward to S- stimuli.

This protocol combines the procedures followed at the two marmoset testing sites of the TRIDENT, namely the Western and McGill sites, into a single, unified protocol.

MATERIALS

Apparatus:

- i. **McGill:** In-cage touchscreen testing Non-human Primate (NHP) Smart Chair (designed by Rogue Research Inc), with a face recognition USB industrial camera.
- ii. **Western:** In-cage touchscreen testing using Raspberry Pi-based Marmtouch software





Western Touch Screen Box

Software:

Both sites use the below softwares:

- i. MATLAB and MonkeyLogic (customized version 2.2.32)
- ii. Python and R (for analyses)

PROCEDURES

1.GENERAL HARDWARE CHECK

- i. The hardware is quickly tested prior to every training or testing session to ensure the program is running properly. This must be done before the marmoset is in the box/chair to prevent returning the marmoset without training due to technical difficulties.
- ii. The items to check include:
 - 1) the NHP Smart Chair/Touchscreen box is attached to the home cage,
 - 2) the reward pump delivers the reward,
 - 3) the touchscreen is sensitive and responding to the touches, and
 - 4) the task shows expected outputs (visual stimuli are correctly displayed, and reward is correctly delivered) and inputs (touching the correct stimulus triggers the reward, touching the incorrect stimulus triggers the punishment – longer ITI).
- iii. Training/testing schedule:

Western Site: Marmosets perform one training session per day at the Western site, usually in the morning, before the feeding routine.

McGill Site: Since the training is self-initiated, a single session can often be split with breaks in between as the marmosets return to the cage before completing a set limit of trials. However, the total number of trials are limited to 30. Marmosets perform one training session per day, usually before their afternoon feeding to maximize performance. Further, the schedule is subject to change based on the animal health care team's health check and husbandry schedule.

2. PRE-TRAINING PROCEDURES

Phase 1: Acclimation

a. Aim:

Acclimation allows marmosets to habituate to the new apparatus and to enter the Smart Chair or the touchscreen box. And further allows marmosets to associate touching the screen with acquiring a reward from the delivery pump.

Note: Exhibit caution and do not allow marmosets to sit idle and do nothing in the box. The marmosets need to associate the box/chair to doing the task (work) to get their reward.

b. Criteria:

- i. Marmosets spend approximately 45 minutes in the Smart Chair or touchscreen box. Marmosets seem calm and motivated to remain in the chair or box. A criterion for progressing to the next phase is that the marmoset remains in the box to consume the reward, rather than running back and forth to the home cage, as such behaviour may indicate fear or discomfort with the testing environment.
- ii. Marmosets complete 30 trials within 45 minutes. At McGill site, marmosets are given 1 hour 30 minutes for self-initiated access to complete the trials.

c. Instructions:

- i. The Smart Chair/Touchscreen box is attached to the marmoset home cage.
- ii. Exploratory behavior is encouraged by sticking marshmallow pieces on the screen and enrichment objects in the box.
- iii. Display a visual stimulus on a black background centered on the screen. Visual stimulus must be something attractive for the marmoset (marmoset face, pictures of marmosets grooming/fighting, food).
- iv. The marshmallows on the screen attract the monkey. Reward marmosets for touching the presented stimuli. At McGill site, there is a random 30% chance of a bonus for correct response to help with conditional learning.
- v. Inter-trial interval (ITI) set at 5 seconds.
- vi. To avoid distressing the marmosets, the door to the Smart Chair/Touchscreen box is not closed. This allows the marmosets to freely move in and out of the Smart Chair/Touchscreen Box.

Phase 2: Unguided Touch-Reward Association**a. Aim:**

For marmosets to associate touching the screen with acquiring a reward from the delivery pump.

b. Criteria:

Marmosets complete 30 trials of successful touch and reward acquisition within 45 minutes.

c. Instructions:

- i. The Smart Chair/Touchscreen box is attached to the marmoset home cage.
- ii. The same task and stimuli of the previous phase are presented to the marmoset.
- iii. Enrichment objects and marshmallows on the screen are gradually removed from the chair/box, until there are no objects or marshmallows left. The aim is to have them touch the screen autonomously, without a marshmallow as a guide.
- iv. **(Cage/marmoset dependent)** Only a small amount of fluff (reward) will be placed on the screen at the beginning of the training as motivation, thus the first 5 trials will be removed from analysis due to this.

Phase 3: Locational Touchscreen Learning**a. Aim:**

Marmosets should learn to associate the visual stimuli with a reward by touching different and specific parts of the touchscreen where the visual stimuli are displayed.

b. Criteria:

Marmosets complete 30 trials of successful touch and reward acquisition within 45 minutes.

c. Instructions:

- i. The Smart Chair/Touchscreen box is attached to the marmoset home cage.
- ii. A new task is presented, displaying two squared stimuli (100x100 pixels) on the right and left sides of the screen. Stimuli do not need to be attractive per se: the marmosets now know by touching the screen they can obtain the reward.

d. Workflow of the task:

- i. Reward marmosets for touching one of the presented stimuli (both stimuli represent a correct response). 30% chance of a bonus (double reward).
- ii. Inter-trial interval (ITI) at 5 seconds.
- iii. Time-out of 2 minutes: if the marmoset does not touch the stimuli before the time-out, the ITI is triggered and a next trial begins.

Phase 4: Pairwise Visual Discrimination Learning (Discrimination Learning)***a. Aim:***

For marmosets to learn which of the two stimuli is rewarding by improving their accuracy of associating rewarded stimuli with more training.

b. Criteria:

90% accuracy (namely 27 correct responses out of 30 trials presented; or any other desired level of accuracy but no lower than 80%; a response is considered correct only if the marmoset touches the S+ stimulus) over 2 consecutive days of training, with 60 minutes to 90 minutes for 3 marmosets (30 to 60 minutes per marmoset for Western, depending on the animal needs) training time per day.

c. Instructions:

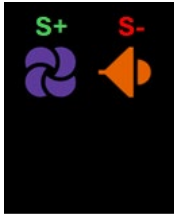
- i. The Smart Chair/Touchscreen box is attached to the marmoset home cage.
- ii. Launch the PVD task
 - Tap on the Marmtouch icon
 - On the right-side menu, tap on Tasks
 - Select the task (Reversal)
 - Select the monkey (stimuli pairs can be monkey-specific)

d. Workflow of the task:

- i. At Western, the animal identity that is performing the task is pre-determined before they enter the box. At McGill, a camera will be placed near the touchscreen. When marmosets enter, their identity will be recognized using a face recognition program. The PVD task will be launched based on individual's progress.
- ii. After scanning, two high-contrast PVD stimuli will be presented on the touchscreen simultaneously on either side of the screen (100x100 pixels). One of the stimuli will be rewarding (S+), and one will not be (S-). When

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touching the S+ stimuli, the marmosets will be given 0.075 mL of the reward (increasable if specific marmosets need it).



- iii. After the selection of stimuli, the correct response will persist on the screen for 1 second, while the incorrect response disappears. This fosters the animal's attention on the correct stimulus.
- iv. As marmosets at McGill have free access to the touchscreen during the task, the ITI at McGill is shorter to maintain the focus of the marmosets. The default ITI is 2 seconds (4 seconds at Western). For incorrect responses, the ITI increases to 5 seconds (10 seconds at Western).

Phase 5: Pairwise Visual Discrimination Reversal (Reversal Learning)

a. Aim:

For marmosets to learn which of the two stimuli is rewarding after a reversal. This task aims to investigate the cognitive flexibility of the common marmoset.

b. Criteria:

90% accuracy over 2 consecutive days of training, (30 to 60 minutes per marmoset for Western, 60 to 90 minutes for 3 marmosets for McGill) training time per day.

c. Instructions:

- i. The Smart Chair/Touchscreen box is attached to the marmoset home cage.
- ii. The PVD task is launched, after reversing the association between visual stimuli and reward.

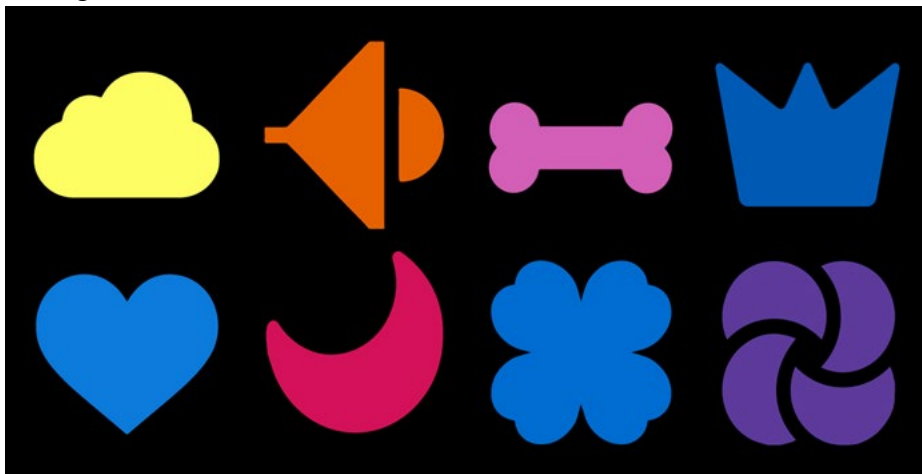
d. Workflow of the task:

- ii. The task procedures are identical to **the previous PVD learning phase**. However, the contingency of reward association of the 2 PVD stimuli will be reversed: the stimulus previously rewarded (S+) will not be rewarded anymore, while the previously unrewarded stimulus (S-) will now be rewarded.

3.DATA COLLECTION PROCEDURES

Phase 6: Baseline and beyond

- a. After completing phases 4 and 5, the marmosets must have learned the PVD task with both the learning and reversal phases. Thus, they are considered ready to proceed with the data collection.
- b. Each data collection PVD session consists of a Discrimination Learning and Reversal Learning phase, using the same pair of stimuli, with the stimulus-reward association reversed during the reversal phase.
- c. Between each PVD cycle, the pair of stimuli will be changed to prevent repetitive stimuli that may confuse the marmosets. The stimuli are shown in the figure below.



- d. The criteria, instruction, and workflow of the task during data collection will be identical to **phases 4 and 5 of the PVD training**.

General Notes:

- The strategy of putting the reward on the touchscreen aims to keep the motivation of the marmosets, which will not be used to guide their choices during the touchscreen PVD task. It is cage/marmoset dependent and not applied on every cage/marmoset that performs the PVD task.
- Do not allow marmosets to be in the smart chair/touchscreen box if the touchscreen is not responsive or if the reward is not dispensing properly.
- If marmosets seem resistant to the initial training phases, you may initially reduce the ITI to encourage marmosets to participate in the tasks. E.g., an ITI of 2 seconds instead of 5 seconds, and then slowly increase the ITI until it reaches the prescribed criteria. For some animals, 4 seconds ITI can be used as final ITI, in order to foster their task engagement.
 - Steps: 2s, 3s, 4s, 5s

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- If marmosets seem to struggle moving to the next phase of the training, additional, “halfway” tasks may be employed to bridge between phases. Moreover, colorful stimuli can be used to attract marmosets during the tasks. However, all adaptations and changes of the PVD training and task need to be added to this current protocol.
- Maple syrup juice reward:
 - 35g of maple syrup.
 - 1:7 ratio of maple syrup to water (by volume).
- Marshmallow fluff reward:
 - 15g of marshmallow fluff.
 - 60g of water to mix well with the fluff.

Contributed by Dr. Alessandro Zanini (Everling Lab), Jiayue Yang (Clery Lab), and compiled by Dr. Sriram Jayabal (Research Program Director-TRIDENT).



Marmoset Core SOPs

Marmoset Standard Operating Procedures

Title: Protocol for Monitoring Marmoset Daily and Nightly Motor Activity Using NanoWatch

SOP No.: TRIDENT_RP_marmNanoWatch-V1

Date: 29 October 2025

Date last updated: 25 February 2026

Created: For TRIDENT, by Dr. Alessandro Zanini (Western site, Everling Lab), and Jiayue Yang (McGill site, Clery Lab). Protocol comparison by Sriram Jayabal, Research Program Director, TRIDENT.

Protocol for Monitoring Marmoset Daily and Nightly Motor Activity Using NanoWatch

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1. Western Site

1.1 Materials and equipment

- Camntech Nano veterinary actigraph (<https://www.camntech.com/nano/>)
- Camntech MotionWare software
- Motion sensitive camera (or equivalent) for offline reviewing

1.2 Procedures:

1.2.1. Recording Schedule

- **Frequency:** Motor activity is recorded once every ~2 months.
- **Start Time:** NanoWatch is placed on the marmoset on Friday evening around (max) 5 PM. Recording begins at 6 PM. Lights turn off at 7 PM every night.
- **End Time:** NanoWatch is removed on Monday morning, after 9 AM. Lights turn on at 7 AM, every morning.
- **Rationale:** Weekend recordings ensure minimal external disturbances as no other activities (e.g., scanning, touchscreen tasks) are scheduled, apart from feeding routines.

1.2.2. Equipment Setup

- **NanoWatch Holder:** NanoWatch is secured in a holder specifically designed for this purpose (see images).



- **Attachment:** The holder is attached to the head post of the marmoset, which is surgically implanted before baseline measurements.

1.2.3. Baseline Recording

- **Timing:** The first (baseline) recording is conducted 4 to 5 weeks after surgery, to allow the wound margins to heal and ensure the head post is stable.
- **Post-Surgery Consideration:** Surgery involves the implantation of the head post: ensure the monkey is fully healed before the first NanoWatch attachment.

1.2.4. Subsequent Recordings

After the baseline session, subsequent recordings occur at the following time points:

- 10-14 weeks post-surgery
- 18-22 weeks post-surgery
- 26-30 weeks post-surgery
- And so on, once every ~8 weeks

1.2.5. Video Monitoring for Visual Cross-Reference

- **Camera Setup:** A motion-sensitive camera is positioned in front of one of the cages to capture the marmosets' nightly movements.
- **Purpose:** Video footage provides a visual reference to cross-check the motor activity recorded by the NanoWatch. These recordings can be reviewed offline for visual confirmation of the activity patterns.

1.2.6. Room Activity During Recording

- **Minimizing Disturbance:** The weekend was chosen for recordings as there is reduced human activity in the room, limiting external variables that could affect motor activity data.
- **Personnel:** Only personnel responsible for feeding the animals are present during these recordings, ensuring consistency across time points.

Contributed by Dr. Alessandro Zanini (Everling Lab).

2. McGill Site

2.1 Materials and equipment

- Camntech Nano veterinary actigraph (<https://www.camntech.com/nano/>)
- Camntech MotionWare software
- Monitoring camera (or equivalent with infrared night vision) for motion detection or continuous recordings

2.2 Procedures:

2.2.1. **Recording Schedule**

- **Frequency:** Motor activity is recorded once every ~2 months.
- **Start Time:** NanoWatch is placed on the marmoset collar around noon in the middle of the week, so collar length is ensured suitable with 2-3 observation days before weekend. Recording begins at 30 minutes – 1 hour after the placement of NanoWatch. Lights turn off at 8 PM every night.
- **End Time:** NanoWatch is removed on 7 days after the placement. Lights turn on at 8 AM, every morning.
- **Rationale:** 7-day recording capture daily variations in activity and sleep, including differences between weekdays and weekends. Weekend recordings are also included to ensure minimal external disturbances (only AM/PM feeding). No experiments (e.g., scanning, touchscreen tasks) are scheduled during the recording period, apart from feeding routines.

2.2.2. **Equipment Setup - Collar placement**

NanoWatch is attached to the marmosets' collar. For each recording, we hand-hold them for placing and removing the device to their collar (see picture below).



2.2.3. **Experimental timeline**

2.2.3.1. **Baseline Recording Timing**

The first (baseline) recording is conducted 1 week before the PFF injection and stereotaxic surgery, with the NanoWatch removed before the start of the surgery.

2.2.3.2. **Subsequent Recordings**

After the baseline session, subsequent longitudinal recordings occur at the following time points, once every 2 months, one week after the MRI acquisition at the said time point.

2.2.4. Video Monitoring for Visual Cross-Reference**2.2.4.1. Camera Setup**

A top-down motion-detection camera is positioned to the cages, with infrared night vision, to capture the marmosets' movements overnight.

2.2.4.2. Purpose

As NanoWatch is placed on marmosets' collar, video recordings provide additional information about night motions that may be missed by the NanoWatch, especially minimal head movements. These recordings are collected from 11 p.m. to 4 a.m. over 2 days in the weekend. In addition to the embedded motion sensation program in the camera, these videos are applied with motion detection of the activity patterns.

2.2.5. Room Activity During Recording**2.2.5.1. Weekdays**

No training scheduled over the weekdays of the marmoset being recorded, including touchscreen, MRI scanning, etc. Experiments that include taking the marmoset being recorded out from their home cages are not conducted either. However, other procedure may occur in the marmoset housing room concerning other marmosets that can impact the behavior of the recorded marmosets.

2.2.5.2. Personnel

Only lab personnels that marmosets are familiar with, and that are responsible for feeding are present during the recording.

2.2.5.3. Weekends:

Regularly only feeding personnel will be present for no more than 1 hour per day during the weekend (minimal human activities). Thus, overnight motion detection videos are collected during this period across all recording sessions.

2.2.5.4. Personnel

Only personnels performing AM/PM feedings are present during this period.

Contributed by Jiayue Yang (Cléry Lab).

3. Comparison of Western and McGill Marmoset Actigraphy Protocols

3.1 Overview

Both Western and McGill sites use CamNtech Nano veterinary actigraphs and MotionWare software to record motor activity and sleep–wake cycles in marmosets. However, they differ in mounting method, recording duration, timing relative to surgery, and environmental controls.

3.2 Key Similarities

Aspect	Western	McGill
Equipment	CamNtech Nano veterinary actigraph, MotionWare software, camera for motion detection	CamNtech Nano veterinary actigraph, MotionWare, motion-detecting camera with infrared
Frequency	Every ~2 months	Every ~2 months
Rationale	Conducted during periods of minimal disturbance, i.e. beginning Friday evening and ending Monday morning	Includes weekdays and weekends but emphasizes reduced weekend disturbance
Video Cross-reference	Motion-sensitive camera used for visual verification	Infrared motion camera used to detect movements, especially subtle ones missed by NanoWatch
Personnel Limitation	Only feeding staff present	Only familiar feeding staff present during recordings
Goal	Assess motor activity longitudinally in consistent, low-disturbance conditions	Same goal, with additional weekday vs. weekend activity assessment

3.3 Major Differences

3.3.1. Recording Schedule and Duration

Western: Duration ~2.5 days (Friday 6 PM to Monday 9 AM). Weekend-only to minimize disturbance. Lights off at 7 PM, on at 7 AM.

McGill: 7 consecutive days (weekday + weekend). Captures daily variation. Lights off at 8 PM, on at 8 AM.

3.3.2. Device Attachment Method

Western: NanoWatch secured to a surgically implanted head post (requires 4–5 weeks healing). Stable, minimal movement interference.

McGill: NanoWatch attached to collar. Allows pre-surgery baseline recording and easier repeated placement.

3.3.3. Timeline Relative to Surgery

Western: Baseline recording 4–5 weeks post-surgery after healing. Subsequent every 8 weeks.

McGill: Baseline 1 week pre-surgery; subsequent every 2 months, one week after MRI.

3.3.4. Environmental Control and Monitoring

Western: Conducted over quiet weekends, minimal room activity, frontal camera.

McGill: Includes weekdays, top-down infrared camera, weekend videos from 11 PM–4 AM.

3.3.5. Duration and Data Depth

Western: Shorter (~60 hours), highly controlled.

McGill: Longer (7-day continuous), broader variability.

3.3.6. Practical and Conceptual Summary

Dimension	Western (Everling Lab)	McGill (Cléry Lab)
Attachment site	Head post	Collar
Start relative to surgery	4–5 weeks post-surgery	1 week pre-surgery
Duration	~2.5 days	7 days
Timing	Friday evening – Monday morning	Midweek to midweek
Light cycle	7 PM–7 AM	8 PM–8 AM
Noise/Disturbance control	Maximal (weekend only)	Moderate (includes weekdays)
Video angle & duration	Frontal view, overnight	Top-down infrared, 11 PM–4 AM
Goal emphasis	Controlled, stable recovery monitoring	Broader circadian and behavioral profiling

Comparison of the protocols was done by Sriram Jayabal, the Research Program Director-TRIDENT.

Marmoset Standard Operating Procedures

Title: Immunofluorescence Staining on Marmoset Brain Sections

SOP No.: TRIDENT_RP_marmIFS-V1

Date: 22 October 2025

Date last updated: 25 February 2026

Created for TRIDENT, by Jesleen Saini (Western Site, Everling Lab), and compiled by Dr. Sriram Jayabal (Research Program Director, TRIDENT).

Protocol for Immunofluorescence Staining on Marmoset Brain Sections

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1. Introduction

This SOP outlines the standardized immunofluorescence staining procedure for cryosectioned and free-floating marmoset brain sections within the TRIDENT Marmoset Core. The protocol ensures consistent section preparation, antigen retrieval, blocking, antibody incubation, and imaging across sites. Sections are mounted, dried, stained under humidified conditions, and coverslipped for fluorescence microscopy.

2. Materials

- SuperFrost Plus slides
- 0.5X PBS or 1X PBS diluted with distilled water
- Paintbrush for section transfer
- Humidified chamber
- Hydrophobic barrier pen
- TBS 1X
- TBST 1X (TBS + 0.2% Triton X-100)
- Blocking reagents: 5% donkey serum, 2% normal goat serum, or 5% BSA
- Primary and secondary antibodies (listed below)
- Hoechst
- Immuno-mount
- Microscope for visualization

3. Reagents

Citrate Buffer (pH 6.0):

- 10 mM Sodium citrate (~1.47 g in 500 mL)
- Adjust pH to 6.0 using 12M HCl
- Add 250 μ L Tween-20 to achieve 0.05% concentration

1X TBST (TBS + 0.2% Triton-X-100):

- Add 100 mL 10X TBS to 900 mL distilled water
- Add 2 mL Triton X-100 and mix thoroughly

4. Primary and Secondary Antibodies

Primary Antibodies:

- pS129 (Abcam ab51253)
- Iba1 (SYSY 234009)

Secondary Antibodies:

- Donkey Anti-Rabbit Alexa 647 (for pS129)
- Donkey Anti-Chicken Alexa 488 (for Iba1)
- Hoechst for nuclear labeling

5. Brain Slicing Scheme

All slices from the whole brain are collected and stored for immediate use, and for potential re-staining in the future.

For immunostaining, slices of interest are typically picked at +17.0mm, +15.0mm, +13.0 mm, +12.5 mm, +10 mm, +11.0 mm, +9.5 mm, +8.5 mm, +6.5 mm, +4.5 mm, +2.5 mm, 0.0 mm, – 2.0mm, and – 4.0mm from the interneural line. After reaching the interneural line, sections are stained every 2 mm throughout the posterior brain. This scheme allows for a comprehensive assessment of biomarkers through the brain.

6. Procedures

1. **Free-Floating Sections:** Using a Petri dish, mount sections using 0.5X PBS (you can use 1X PBS plus distilled water, the distilled water helps especially).
 - a. Mount sections onto SuperFrost Plus slides (this will guarantee they stick). Guide sections onto the slide using a paintbrush.
 - b. Let dry for at least 30 minutes to 1 hour, or until sections look completely dry with no moisture left.
2. Add 95°C citrate buffer pH 6 to slides for 20 minutes.

3. Let slides cool down to room temperature (approximately 30 to 40 minutes) on ice in the same buffer.

All following steps are performed in a humid chamber with a hydrophobic barrier drawn around sections:

4. Wash 1 × 10 minutes in TBS 1X.
5. Wash 3 × 10 minutes in TBST 1X (TBS plus 0.2% Triton).
6. Block sections for 1 hour with diluted normal serum (5 percent donkey serum, 2 percent normal goat serum in TBST, from the species in which the secondary antibody is made) or 5 percent BSA.
7. Incubate sections with the primary antibody in blocking buffer (5 percent donkey serum, 2 percent normal goat serum in TBST) or 5 percent BSA, overnight at 4°C.
 - Ps129 - 1:600 dilution
 - Iba1 - 1:500 dilution
8. Wash slides 3 × 10 minutes in TBST 1X.
9. Incubate sections for 2 hours with secondary antibody at room temperature (1:1000 dilution).
10. Wash slides 3 × 10 minutes in TBST 1X.
11. Incubate 10 minutes in Hoechst (1:1000 in TBS 1X).
12. Wash 2 × 10 minutes in TBS 1X.
13. Coverslip with Immuno-Mount.
14. Visualize on microscope.

7. Additional Notes

- Perfusion is typically performed immediately after the final imaging session. In unavoidable circumstances, provided animal health remains stable, a delay is acceptable, but the timing should be recorded.
- Post-perfusion, the head is stored in PFA for 1 week, followed by PBSN (PBS with sodium azide) for another week before brain extraction.
- Extracted brains may be stored long-term in PBSN. Replace PBSN every 2 months or sooner if the storage medium turns cloudy or odorous.

Contributed by Jesleen Saini (Everling Lab).

Marmoset Standard Operating Procedures

Title: MRI imaging of live awake marmosets – Anatomical and functional imaging
SOP

SOP No.: TRIDENT_RP_marmMRI-V1

Date: 20 Jan 2026

Date last updated: 25 February 2026

Created for TRIDENT, by Alessandro Zanini (Western Site, Everling Lab),
Jiayue Yang (McGill Site, Clery Lab) and compiled by Dr. Sriram Jayabal
(Research Program Director, TRIDENT).

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Introduction

This SOP describes the standardized procedure for functional magnetic resonance imaging (fMRI) in awake common marmosets (*Callithrix jacchus*) using a 9.4T scanner at Western University and a 7T scanner at McGill University. It covers MRI training, animal positioning, physiological monitoring, and imaging acquisition.

Western Site

1. Materials and Equipment

1.1. fMRI Setup

- **Scanner:** 9.4 Tesla, 31-cm horizontal bore magnet (Bruker BioSpec Avance III HD, Paravision 7).
- **Gradient Coil:** Custom-built, 15-cm inner diameter, maximum gradient strength 430 millitesla per meter (mT/m).
- **Receive System:** Eight receive channels with Siemens preamplifiers, connected to an in-house built quadrature birdcage coil (12-cm inner diameter, used for radiofrequency transmission).

1.1.1. Functional Imaging Parameters

Parameter	Value	Notes
Sequence	Gradient-echo, single-shot Echo Planar Imaging (EPI)	High temporal resolution for BOLD fMRI
Repetition Time (TR)	1.5 seconds	Matches TR in continuous acquisitions
Acquisition Time (TA)	1.5 seconds	Matches TR in continuous acquisitions
Echo Time (TE)	15 milliseconds	Optimized for BOLD contrast
Flip Angle	40°	Standard for minimizing SAR while maintaining signal
Field of View (FOV)	64 × 48 mm ²	Covers marmoset brain
Matrix Size	96 × 128	Produces isotropic resolution
Voxel Size	0.5 mm isotropic	Balance between SNR and spatial resolution
Number of Slices	42 (axial orientation)	Whole-brain coverage

Bandwidth	400 kilohertz	Minimizes distortion, supports short TE
Parallel Imaging	GRAPPA factor = 2 (left-right direction)	Reduces acquisition time

1.1.2 Distortion Correction

- Step: Acquire an additional set of EPI scans with the opposite phase-encoding direction (right-to-left instead of left-to-right).
- Rationale: EPI sequences are sensitive to spatial distortions caused by magnetic field inhomogeneities (B0). Reversed phase-encoding images allow estimation and correction of these distortions during preprocessing, ensuring accurate alignment of functional and anatomical images.

1.1.3 Anatomical Imaging Parameters

Parameter	Value	Notes
Sequence	T2-weighted structural imaging	Provides anatomical reference
Repetition Time (TR)	7 seconds	Long TR for T2-weighting
Echo Time (TE)	52 milliseconds	Contrast optimized for anatomy
Field of View (FOV)	51.2 × 51.2 mm ²	Focused on brain structures
Voxel Size	0.133 × 0.133 × 0.5 mm ³	High-resolution structural detail
Number of Slices	45 (axial orientation)	Whole-brain coverage
Bandwidth	50 kilohertz	Lower bandwidth allows higher SNR
Parallel Imaging	GRAPPA factor = 2	Shortens acquisition time

1.2. Physiological Monitoring Setup

- Video Monitoring: MRI-compatible camera (60 Hz, MRC Systems 12M-i) positioned in front of the animal, connected to an external screen for real-time observation of wakefulness and motor agitation.
- Respiratory Monitoring: The experimenter regularly monitors respiration. Depending on the set up, a respiratory pillow connected to LabChart software (SA Instruments) can be used to continuously monitor breathing rate and rhythm.
- Personnel: Veterinary technician and/or the experimenter must monitor video and respiratory signals throughout each scanning session.

1.3. Animal Safety Considerations

1.3.1 Magnetic Field Sensitivity

- Marmosets may exhibit transient nausea or nystagmus upon initial exposure to the magnetic field.
- To minimize symptoms, an automated bed is used to insert the body restraint slowly into the scanner bore to allow gradual adaptation before acquisitions begin. This automation allows for constant low speed, and allows for a more standardized approach across animals.

1.3.2 Critical Warning: Vomiting Risk

- Signs of nausea: swallowing, licking, repeated vomiting.
- Risk: Aspiration due to prone, head-fixed position, which can obstruct breathing.
- Actions:
 1. Continuously monitor video feed.
 2. If vomiting is repeated, or prolonged motor agitation is observed, remove the animal immediately.
 3. The automated bed is used for retrieval of the animal under distress should need be.

2. Procedure

2.1 Surgery

A headpost needs to be affixed to restrain the marmoset in the body restraint system using the PEEK headpost (**Figure 1**) while imaging. Please refer to the

Surgery protocol for PFF injection SOP, which also describes the affixation of the headpost.

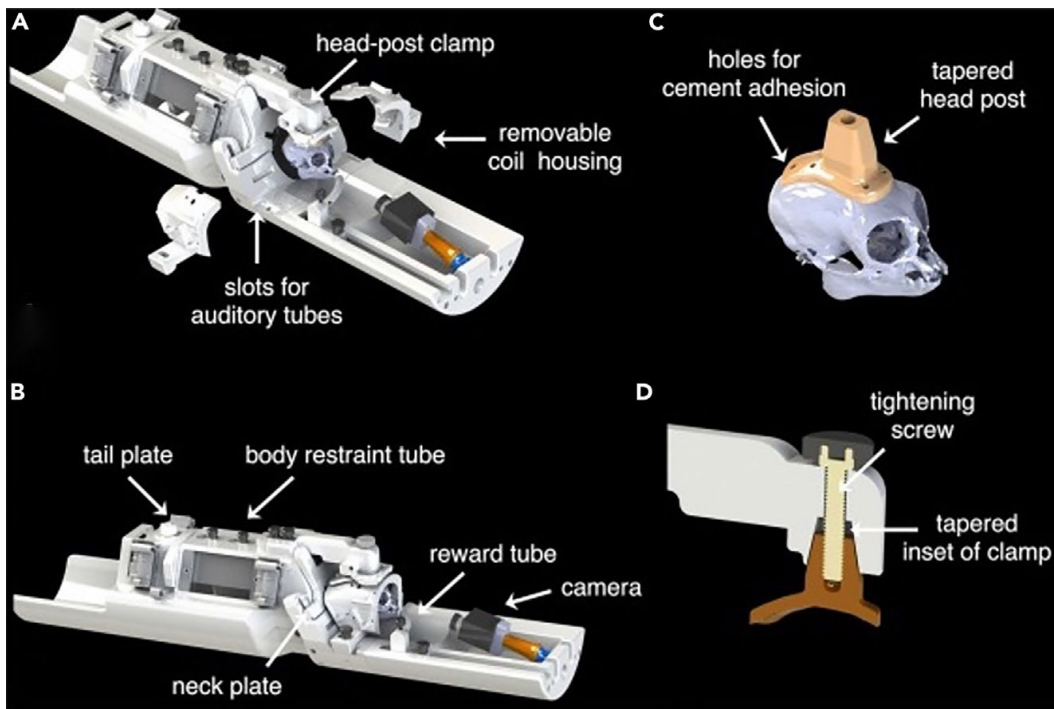


Figure 1. Detailed representation of the body restraint system and the PEEK headpost

(A) CAD drawing of the body restraint. Sequence of securing the marmoset: neck plate, back plate, tail plate, head-post clamp (with two or three anchoring points to the body restraint depending on the system being used, see Figure 4 for an example of a three-points anchoring method), and ear protection (earphones or silicone earplugs).

(B) Coil housing: attachment of the two coil parts to the front slots of the body restraint.

(C) Position and size of the implanted head post on the marmoset skull.

(D) Fixation procedure: securing the head post into the clamp with a PEEK screw, then attaching the clamp to the body restraint.

2.2. MRI Training (3 Weeks)

Week 1: Body Restraint Acclimatization

1. Place the marmoset inside the body restraint tube without head fixation (**Figure 2**).
 - Allow the animal to enter from the rear opening until its head emerges from the anterior hole.
 - Secure the animal by lowering the neck plate.
 - Insert an appropriately sized back plate above the animal's back to fill dead space and reduce movement.
2. Begin with 10-minute sessions and gradually extend to 30 minutes.
3. No additional stimuli are presented during this stage.
 - **Note:** Restraint design was 3D-printed in-house; CAD files are publicly available (see resources).

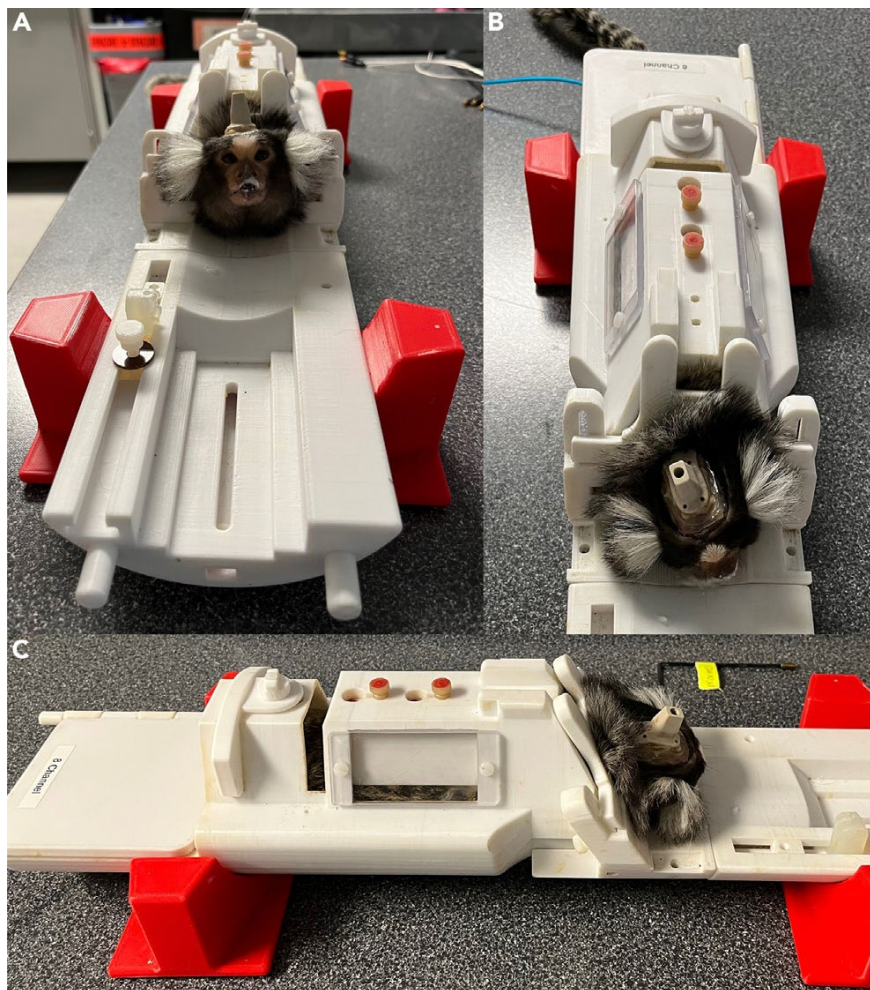


Figure 2. First week of MRI training: Familiarization with the body restraint

(A) Frontal view of the training setup during Week 1.

(B) Superior view of the training setup during Week 1.

(C) Lateral view of the training setup during Week 1.

Note: In this stage, the marmoset's head is not fixed to the body restraint, and no gradient coil sounds.

Week 2: Mock MRI Tube Acclimatization

1. Place the marmoset in the body restraint, then insert the restraint into a **mock MRI tube** (e.g., plexiglass tube large enough to fit the animal holder).
2. Do not fixate the head at this stage.
3. Introduce scanner-like acoustic noise by playing gradient-coil recordings.
 - Gradually increase volume up to **80 dB** (lower than actual 9.4 T MRI noise, but sufficient for training).
 - Noise during real scans will be further reduced with silicone earplugs.
4. Increase training duration in **10-minute increments**, up to **60 minutes**.

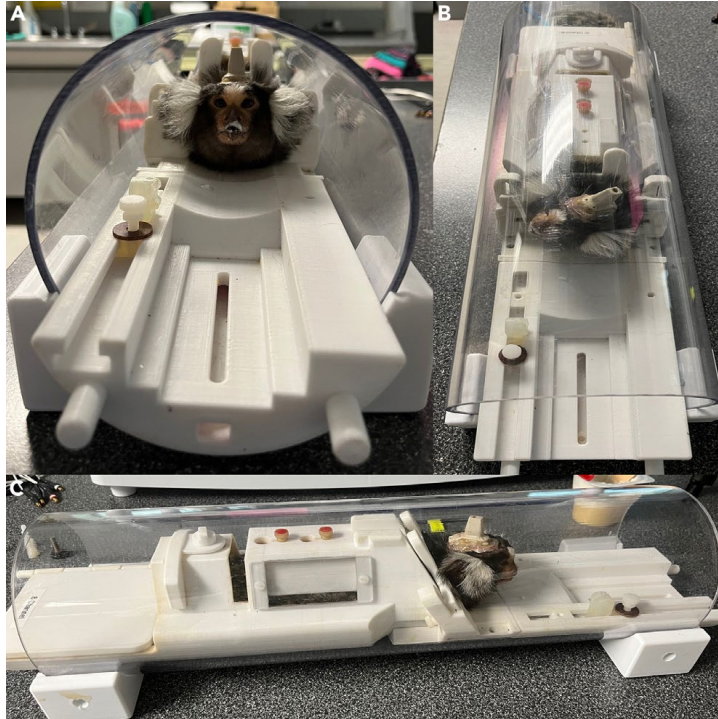


Figure 3. Second week of MRI training: Gradient-coil sounds in the mock-MRI scanner

(A) Frontal view of the training setup during Week 2.

(B) Superior view of the training setup during Week 2.

(C) Lateral view of the training setup during Week 2.

Note: In this stage, the marmoset's head is not fixed, but the body restraint is positioned inside a mock-MRI scanner and gradient-coil sounds are played.

Week 3: Head Fixation Acclimatization

1. Repeat steps from Weeks 1 and 2.
2. Fix the marmoset's head post into the appropriate clamp for the first time.
 - Ensure the head post is secured in the same position used during actual MRI sessions.
3. Continue gradually increasing session duration (to a maximum of 90 min) as tolerated by the animal.



Figure 4. Third week of MRI training: Head fixation

(A) Frontal view of the training setup during Week 3.

(B) Superior view of the training setup during Week 3.

(C) Lateral view of the training setup during Week 3.

Note: In this stage, the marmoset's head is fixed to the body restraint using the head-post clamp. The body restraint is placed inside a mock-MRI tube and gradient-coil sounds are played.

Critical Notes

- **Progression Criteria:** Only advance to the next training stage when the animal displays calm behavior (no or limited body movements, no excessive vocalizations, no signs of excessive anxiety).
- **Positive Reinforcement:** Provide small food rewards (e.g., pudding or marshmallow fluff) to encourage forward orientation and reduce motion.

- **Behavioral Assessment:** Training is considered successful when the animal achieves a score of **1 or 2** on the behavioral assessment scale (Silva et al., ref. 15).
- **Animal Safety:** Training sessions must be supervised, with continuous observation for signs of agitation, distress, or abnormal behavior.

2.3. Marmoset Positioning Before fMRI Session (10–15 min)

Preparation

- Perform all steps in the preparation room (outside scanner room).
- Prepare all necessary materials before the marmoset arrives to minimize stress.
- Ensure the cradle, restraint, coil housing, preamplifiers, and monitoring devices are ready.

Procedure

Step 1: Position in Body Restraint

1. Guide the marmoset headfirst into the body restraint via the rear opening.
 - Position the body restraint on the two supports, in order to elevate and hold it in place while loading the animal.
2. Hold the neck plate open, gently push the animal forward until the head exits the front opening.
3. Close the neck plate and tighten side screws to prevent backing up.
 - **Critical:** Ensure skin on the neck/shoulders is not pinched.

Step 2: Head Fixation

4. Remove the protective screw from the head post.
5. Insert head post into clamp; secure with the PEEK screw.
6. Tighten fixation screws:
 - Four screws (two anterior, two posterior). Eliminates head motion.
 - **Note:** Ensure headpost and all other screws are well tightened

Step 3: Back and Tail Plates

7. Select the back plate that fits the animal size.
 - Check by flattening the back and testing for space.
 - The correct plate prevents arching but does not apply pressure.
8. Secure the back plate with two screws.
9. Close the tail plate.
 - Use posterior slot for larger animals, anterior slot for smaller.
 - Optional: Add folding tail cover to limit tail motion and prevent cable interference.

Step 4: Ear Protection

10. If **no auditory stimuli**, insert silicone earplugs only.

Step 5: Coil Housing

11. Attach and secure the two removable coil housing parts with screws.
 - Ensure front edges are aligned to avoid signal loss (**Figure 5**).

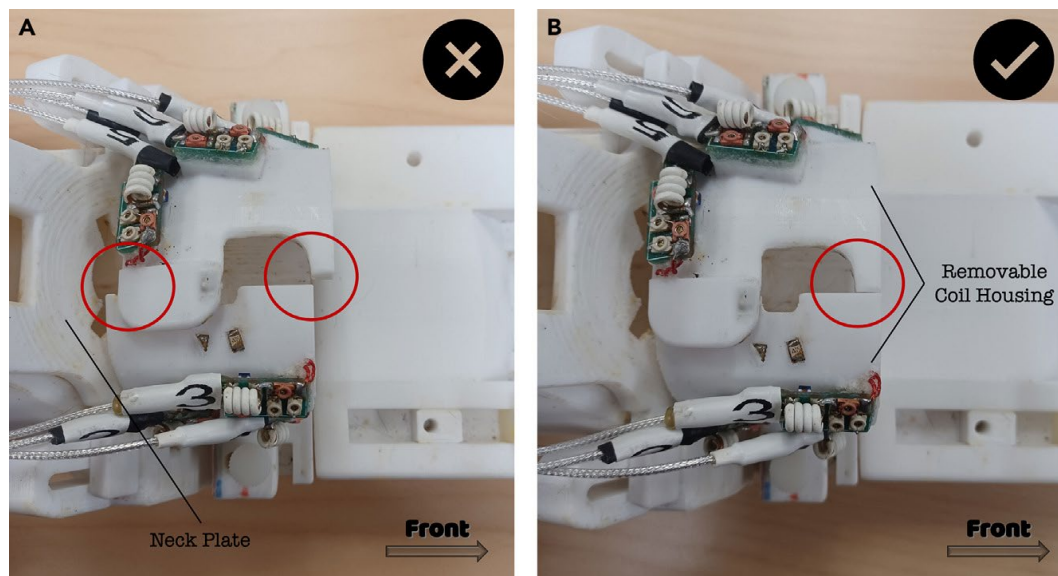


Figure 5. Detailed view of the removable coil housing positioning

(A) Example of incorrect positioning of the two removable coil housing parts (frontal–posterior shift).

(B) Example of correct positioning of the two removable coil housing parts.

Note: Misalignment can negatively affect signal quality during MR acquisition.

Step 6: Final Setup in Cradle

12. Bring the body restraint to the automated bed, positioning it on the cradle.
13. Slide the body restraint forward until the **isocenter mark** aligns with coil housing.
 - Adjust depending on laboratory setup.

Step 7: Electronics and Monitoring

14. Connect the eight coil channels to their connectors.
15. Connect cables for the MRI-compatible camera and respiratory pillow, if any.
16. Verify monitoring:
 - Visual feed from camera.
 - Physiological signal from the respiratory pillow, if any.

Staffing

- Procedure can be done by one trained operator, but **two experimenters are recommended**:
 - **Posterior tasks**: inserting animal, back/tail plates.
 - **Anterior tasks**: neck plate, head fixation, ear protection, coil housing.

2.4. fMRI Acquisition (Approx. 1 hr)

Step 1: Localizer Scan

1. Run a **standard localizer sequence** to identify head and brain position.
2. Confirm that the brain is aligned with the **magnet isocenter**.

Step 2: RF Coil Calibration

3. Calibrate the transmit **radiofrequency (RF) coils** to ensure accurate flip angles and signal consistency.

Step 3: Magnetic Field Shimming

4. Run the **MAPSHIM protocol** to optimize homogeneity of the main magnetic field (B0).
 - **Note:** Accurate placement of the marmoset's head at the isocenter improves shimming efficacy and reduces inter-animal variation in distortion.
 - **Troubleshooting:** If images remain highly distorted or signal is low after shimming, refer to Troubleshooting 3.

Critical Notes

- Proper isocenter positioning is crucial for reducing geometric distortion.
- Insufficient shimming can result in poor image quality and unusable functional data.
- Always review scout/localizer images before proceeding to functional acquisitions.

2.5. Monitoring During Imaging

- Observe animal continuously using MRI-compatible camera.
- Watch for signs of distress (agitation, nausea, excessive movement).
- If vomiting or respiratory compromise occurs, remove animal slowly but promptly from bore.

Troubleshooting**Problem 1: Resistance to Enter Body Restraint****Description**

Some marmosets resist entering the body restraint. Resistance can manifest as:

- Using upper limbs to counter rear pressure.
- Lowering the head to block passage through the anterior opening.
- In smaller animals (~300 g), attempting to turn inside the restraint and exit through the rear.
(Related to Step 5b of Positioning SOP).

Potential Solutions

- Use **two experimenters**:

- One gently pushes the marmoset forward.
- The other keeps the neck plate open and attracts the animal forward with small food rewards (pudding, marshmallows).
- If resistance persists:
 - One experimenter may gently guide the upper limbs or head post to allow proper orientation.
- For small marmosets:
 - Insert a soft, bulky item (e.g., thick handling glove) through the front opening to reduce internal space and prevent turning.
 - The glove also provides something to bite once grabbed, slowly withdraw it while gently pushing forward.

Critical Note

- Never increase rear pressure forcefully to push the head through.
- This risks improper positioning or violent impact of the head post against the restraint, which may cause severe injury due to the thin braincase.

Problem 2: Distorted or Low-Signal Images After Shimming**Description**

Following localizer and shimming, images may appear highly distorted or with very low signal.

(Related to Step 8 of Acquisition SOP).

Potential Solutions

1. **Repeat the shimming** and check for improvements.
2. Inspect setup:
 - Confirm correct positioning of coil housing parts.
 - Verify all receive channels are connected properly to preamplifiers.
3. Check for **animal movement**:
 - Even with restraint and head fixation, some marmosets may move body, limbs, or tail.

- Solutions:
 - Ensure back plate is thick enough to minimize body motion.
 - Reinsert cradle, repeat localizer and shimming.

4. Extend or repeat training if agitation persists:

- Some animals regress under scanner conditions (noise, environment, magnetic field).
- Resume Phase 3 of MRI training outside the scanner, ideally in the preparation room where stress occurs, to reduce agitation.

Hardware Resources

- **3D-Printed Restraint Components:**

- CAD files for the body restraint, PEEK head post, and other printed components are publicly available.
- CAD file for the 3D printed anesthesia mask:
<https://doi.org/10.5281/zenodo.8045220>
- Repository: [OSF – DOI: 10.17605/OSF.IO/VTQEP](https://osf.io/VTQEP).

Documentation

- Record date, animal ID, session duration, stimulation type, and any issues.
- Store imaging parameters and raw data securely on lab server.
- Log animal welfare notes before, during, and after imaging.

Citation

This protocol was adapted from:

Zanini A., Dureux A., Jafari A., Gilbert K.M., Zeman P., Bellyou M., Li A., Vander Tuin C., and Everling S. (2023). In vivo functional brain mapping using ultra-high-field fMRI in awake common marmosets. STAR Protocols 4, 102586.

DOI: <https://doi.org/10.1016/j.xpro.2023.102586>

Video resources for the procedures described above can be found in the Supplemental Information of the article cited. Viewing these videos is highly encouraged to ensure proper understanding and replication of each step.

McGill Site

1. Materials and Equipment

1.1. fMRI Setup

- **Scanner:** 7 Tesla whole body MR system (MAGNETOM Terra, Siemens Healthineers, Erlangen Germany)
- **Transmit Coil:** Shielded 24-rung band-pass birdcage with a quadrature hybrid. The transmit coil has a diameter of 15 cm and 24 legs (21cm length per leg).
- **Receiving Array:** 8-element phased array with varying loop shapes and sizes (2-4 cm loop diameters), the array was built upon a custom, 3D printed marmoset helmet/head former. Each Rx loop was tuned to the ^1H , 7T operating frequency (297.2 MHz) and was built with an active detuning circuit to actively decouple the receive loop during transmission.

1.1.1. Functional Imaging Parameters

Parameter	Value	Notes
Sequence	Gradient-echo, single-shot Echo Planar Imaging (EPI)	High temporal resolution for BOLD fMRI
Repetition Time (TR)	1.5 seconds	Matches TR in continuous acquisitions
Acquisition Time (TA)	1.5 seconds	Matches TR in continuous acquisitions
Echo Time (TE)	17 milliseconds	Optimized for BOLD contrast
Flip Angle	40°	Standard for minimizing SAR while maintaining signal
Field of View (FOV)	50 × 37.5 mm ²	Covers marmoset brain
Matrix Size	64 × 64	Produces isotropic resolution
Voxel Size	0.8 mm isotropic	Balance between SNR and spatial resolution

Number of Slices	30 (coronal orientation)	Whole-brain coverage
Bandwidth	1.30 kHz/pixel	Minimizes distortion, supports short TE
Parallel Imaging	GRAPPA factor = 3 (left–right phase-encoding direction)	Reduces acquisition time

1.1.2 Distortion Correction

- Step: Acquire an additional set of EPI scans with the opposite phase-encoding direction (right-to-left instead of left-to-right).
- Rationale: EPI sequences are sensitive to spatial distortions caused by magnetic field inhomogeneities (B0). Reversed phase-encoding images allow estimation and correction of these distortions during preprocessing, ensuring accurate alignment of functional and anatomical images.

1.1.3 Anatomical Imaging Parameters

Parameter	Value	Notes
Sequence	3D T2-weighted SPACE (SPC)	Provides anatomical reference
Repetition Time (TR)	3 seconds	Long TR for T2-weighting
Echo Time (TE)	515 ms	Contrast optimized for anatomy
Field of View (FOV)	70 × 59.1 mm ²	Focused on brain structures
Voxel Size	0.2 × 0.2 × 0.2 mm ³	High-resolution structural detail
Number of Slices	208 (coronal orientation)	Whole-brain coverage
Bandwidth	0.35 kHz/pixel	Lower bandwidth allows higher SNR

Parallel Imaging

GRAPPA factor = 2
(phase-encoding)

Shortens acquisition
time

1.2. Physiological Monitoring Setup

- Video Monitoring: MRI-compatible camera (Model 12M-i, 60-Hz sampling rate, MRC Systems GmbH, Heidelberg, Germany), attached on a customized camera holder, is positioned in front of the animal and focused on animal eyes, connected to an external screen for real-time observation of wakefulness and motor agitation.
- Visual Fixation Stimulus (optional, recommended for awake imaging): A simple high-contrast fixation target (e.g., centered black dot on a uniform background) may be presented on the in-bore screen facing the animal during scanning to promote stable gaze and reduce eye movements, including nystagmus. Use should be documented in the scan log.
- Personnel: Experimenter monitors video throughout each scanning session.

1.3. Animal Safety Considerations

1.3.1 Magnetic Field Sensitivity

- Marmosets may exhibit transient nausea or nystagmus upon initial exposure to the magnetic field.
- To minimize symptoms, an automated bed is used to insert the body restraint slowly into the scanner bore to allow gradual adaptation before acquisitions begin. This automation allows for constant low speed and allows for a more standardized approach across animals.

1.3.2 Critical Warning: Vomiting Risk

- Signs of nausea: swallowing, licking, repeated vomiting.
- Risk: Aspiration due to prone, head-fixed position, which can obstruct breathing.
- Actions:
 1. Continuously monitor video feed.
 2. If vomiting is repeated, or prolonged motor agitation is observed, remove the animal immediately.
 3. The automated bed is used for retrieval of the animal under distress should

need be.

2. Procedure

2.1 CT scan

An individualized CT scan is acquired to localize the injection site of marmosets. Based on the CT scan, we model the head structure of the marmoset and specifically design an individualized 3D-printed helmet based on their head shape (**Figure 1**). This aims to ensure a fit between the helmet and marmoset head, such that the head motion can be maximally reduced while mounting the helmet on the MRI chair.

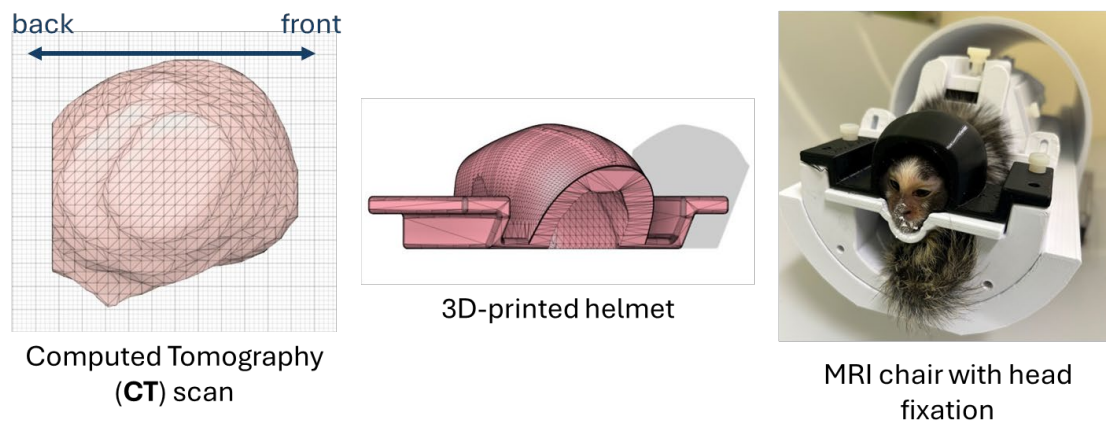


Figure 1. Schematic and example design of the 3D-printed helmet.

(Left) 3D model of the marmoset head shape, extracted from the CT scan.

(Middle) Customized 3D-printed helmet based on the marmoset head shape (example in the left panel).

(Right) Example photo of the marmoset wearing the 3D-printed helmet on the MRI chair.

2.2. MRI Training (3 Weeks)

Week 1: Body Restraint Acclimatization

4. Place the marmoset inside the body restraint tube without head fixation.
 - Allow the animal to enter from the rear opening until its head emerges from the anterior hole.

- Secure the animal by the neck and tail plates (**Figure 2A**).
 - Insert an appropriately sized back plate above the animal's back to fill dead space and reduce movement.
5. Begin with 10-minute sessions and gradually extend to 30 minutes.
 6. No additional stimuli are presented during this stage.
 - **Note:** Restraint design was 3D-printed in-house; CAD files are publicly available (see resources).

Week 2: Mock MRI Tube Acclimatization

5. Place the marmoset in the body restraint, then insert the restraint into a **mock MRI tube** (e.g., plexiglass tube large enough to fit the animal holder) (**Figure 2B**).
6. Do not fixate the head at this stage.
7. Introduce scanner-like acoustic noise by playing gradient-coil recordings.
 - Noises are increased slowly in volumes.
8. Increase training duration in **10-minute increments**, up to **60 minutes**.

Week 3: Head Fixation Acclimatization

4. Repeat steps from Weeks 1 and 2.
5. Fix the marmoset's head post into the appropriate clamp for the first time (**Figure 1, right panel**).
 - Ensure the head post is secured in the same position used during actual MRI sessions.
6. Continue gradually increasing session duration (to a maximum of 90 min) as tolerated by the animal.

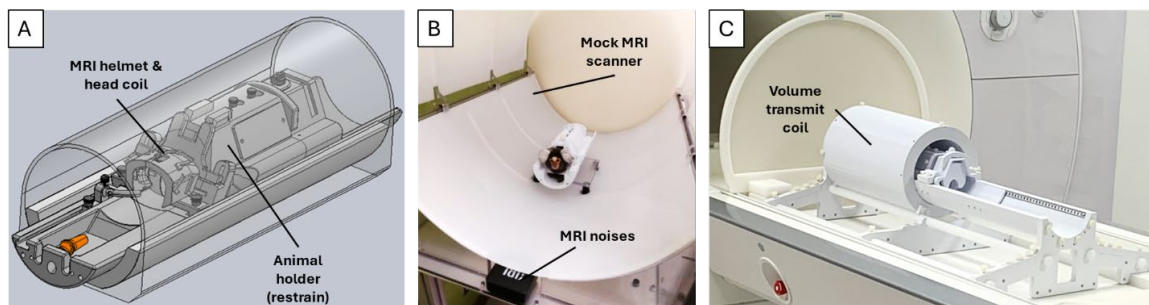


Figure 2. Schematic and example design of the 3D-printed helmet.

(A) The MRI holder, which is designed to hold the animal and restrain their movement during the MRI scan.

(B) Example setup of week 2 acclimation, a marmoset is restrained in the MRI chair, with MRI noises, in the mock MRI scanner.

(C) Experimental setup of the MRI transmit coil and the MRI chair in the 7T scanner.

Critical Notes

- **Progression Criteria:** Only advance to the next training stage when the animal displays calm behavior (no or limited body movements, no excessive vocalizations, no signs of excessive anxiety).
- **Positive Reinforcement:** Provide small food rewards (e.g., yogurt chips or marshmallow fluff) to encourage forward orientation and reduce motion.
- **Behavioral Assessment:** Training is considered successful when the animal achieves a score of **1 or 2** on the behavioral assessment scale (Silva et al., 2011).
- **Animal Safety:** Training sessions must be supervised, with continuous observation for signs of agitation, distress, or abnormal behavior. The helmet must be placed with appropriate tightness, without the risk of causing breathing problem of the animals.

2.3. Marmoset Positioning Before fMRI Session (10–15 min)

Preparation

- Perform all steps, except for the head fixation, in the lab preparation room to reduce stress. Once the animals are secured in the MRI holder, bring them to the preparation room (outside scanner room) using the pet carrier.
- Prepare all necessary materials before the marmoset arrives to minimize stress.
- Ensure the cradle, restraint, coil housing, preamplifiers, and monitoring devices are ready.

Procedure

Step 1: Position in Body Restraint

17. Guide the marmoset headfirst into the body restraint via the rear opening.
 - Position the body restraint on the one support, to transfer from the marmoset transfer box and hold the restraint in place while loading the animal.
18. Hold the neck plate open, gently push the animal forward until the head exits the front opening.
19. Close the neck plate and tighten side screws to prevent backing up.
 - **Critical:** Ensure skin on the neck/shoulders is not pinched.
20. Tighten the back plate and tail plates to reduce animal movement.
 - Check by flattening the back and testing for space.
 - Ensure that the tail and limbs are not pressed or pinched by the plates.
21. Move the animal in a covered transport box (pet carrier) and bring them to the MRI preparation room.

Step 2: Head Fixation

22. Place the chin plate and secure its position with screws.
23. Place the individual-specific MRI helmet on the animal and secure its position with tapes (no screw yet).
 - Make sure that the head is in correct position, with no skin or ear pinched by the helmet placement.
 - Ensure that the animal is breathing well.
24. Tighten fixation screws:
 - Four screws (two anterior, two posterior). Eliminates head motion.
 - **Note:** Ensure all plates are tighten with screws.

Step 6: Final Setup in MRI room

25. Bring the body restraint to the automated bed, positioning it on the cradle (**Figure 2C**).
26. Slide the body restraint forward until the **isocenter mark** aligns with coil housing.
27. Place the MRI coil on the helmet and tighten the screws.
 - Ensure that the screws are not too tight to cause difficulties in breathing.

Step 7: Electronics and Monitoring

28. Connect the eight coil channels to their connectors.
29. Connect cables for the MRI-compatible camera, if any.
30. Secure all MRI coil and camera cables with sandbags to avoid movement by MRI vibrations.
31. Verify monitoring:
 - Visual feed from camera.
 - Physiological signal from the respiratory pillow, if any.

Staffing

- Procedure can be done by one trained operator, but **two experimenters are recommended**:
 - **Posterior tasks**: inserting animal, back/tail plates.
 - **Anterior tasks**: neck plate, head fixation, ear protection, coil housing.

2.4. fMRI Acquisition (Approx. 1 hr)

Step 1: Localizer Scan

3. Run a **standard localizer sequence** to identify head and brain position.
4. Confirm that the brain is aligned with the **magnet isocenter**.

Step 2: RF Coil Calibration

4. Calibrate the transmit **radiofrequency (RF) coils** to ensure accurate flip angles and signal consistency.

Step 3: Magnetic Field Shimming

5. Run the **MAPSHIM protocol** to optimize homogeneity of the main magnetic field (B0).
 - **Note:** Accurate placement of the marmoset's head at the isocenter improves shimming efficacy and reduces inter-animal variation in distortion.
 - **Troubleshooting:** If images remain highly distorted or signal is low after shimming, refer to Troubleshooting 3.

Critical Notes

- Proper isocenter positioning is crucial for reducing geometric distortion.
- Insufficient shimming can result in poor image quality and unusable functional data.
- Always review scout/localizer images before proceeding to functional acquisitions.

2.5. Monitoring During Imaging

- Observe animal continuously using MRI-compatible camera.
- Watch for signs of distress (agitation, nausea, excessive movement).
- If vomiting or respiratory compromise occurs, remove animal slowly but promptly from bore.

Troubleshooting

Problem 1: Resistance to Enter Body Restraint

Description

Some marmosets resist entering the body restraint. Resistance can manifest as:

- Using upper limbs to counter rear pressure.
- Stay in the middle of the restraint and not moving to the neck plate position.
- Enter the restraint reversely (facing the rear opening), refuse to turn around or move to the correct orientation.

Potential Solutions

- If the animal has a **correct orientation (i.e. facing the front opening)**, one experimenter can:
 - Open the neck plate to have more opening space for the marmoset.
 - Gently push the marmoset on the back to encourage them moving forward.
 - Once marmosets in position, slowly drop the neck plate down to secure their position.
- If resistance persists and in reverse orientation (i.e. facing the rear opening). Two experimenters are needed:
 - One experimenter may gently hold the marmoset on the experimenter to allow proper orientation.
 - Another experimenter holds the rear opening of the restraint (without support) to the marmoset.
 - The marmoset then enters the restraint in correct orientation, if there is resistance, gently push them on the back.

Critical Note

- Never increase rear pressure forcefully to push the head through.
- If need to handle the marmoset, make sure that the room doesn't have unknown personnels that may cause the marmoset to be stressed. Handle them gently to avoid potential distress.

Citation

The McGill site protocol was adapted from:

Yazdanbakhsh P, Gacoin M, Couch MJ, Cook T, Leppert IR, Rudko DA, et al. A Birdcage Volume Transmit Coil and 8 Channel Receive Array for Marmoset Brain Imaging at 7T. IEEE transactions on bio-medical engineering [Internet]. 2026;73(1):15–22. Available from: <http://dx.doi.org/10.1109/TBME.2025.3576064>

Silva AC, Liu JV, Hirano Y, Leoni RF, Merkle H, Mackel JB, et al. Longitudinal functional magnetic resonance imaging in animal models. Methods in molecular biology (Clifton, N.J.) [Internet]. 2011;711:281–302. Available from: http://dx.doi.org/10.1007/978-1-61737-992-5_14

Cross-Site Harmonization Summary

1.1 Key Similarities Between Western and McGill MRI Protocols

Category	Shared Feature Across Sites
Animal Model	Awake common marmosets (<i>Callithrix jacchus</i>)
Training Duration	3-week structured acclimatization protocol
Training Structure	Week 1: Body restraint; Week 2: Mock MRI + gradient noise; Week 3: Head fixation
Behavioral Advancement Criteria	Progress only when calm behavior is observed; Silva behavioral score of 1–2 required
Functional Sequence	Gradient-echo, single-shot EPI for BOLD fMRI
Repetition Time (TR)	1.5 seconds
Flip Angle	40 degrees
Distortion Correction Strategy	Reversed phase-encoding EPI scans for geometric distortion correction
Magnetic Field Optimization	MAPSHIM-based B0 shimming protocol
Monitoring During Imaging	Continuous video monitoring throughout scan session
Safety Protocol	Immediate removal if repeated vomiting or prolonged agitation occurs
Head Stabilization Principle	Rigid head fixation to minimize motion artifacts

Parallel Imaging	GRAPPA acceleration used for EPI
Whole-Brain Coverage	Functional and anatomical scans cover entire brain
Data Documentation	Logging of session details, parameters, welfare notes, and secure raw data storage

1.2 Key Differences Between Western and McGill MRI Protocols

Category	Western Site	McGill Site
Scanner Vendor	Bruker BioSpec Avance III HD	Siemens MAGNETOM Terra
Field Strength	9.4 Tesla	7 Tesla
Transmit Coil	In-house quadrature birdcage (12 cm ID)	Shielded 24-rung birdcage (15 cm diameter)
Receive System	8 receive channels with Siemens preamplifiers	8-element phased array on individualized 3D-printed helmet
Head Stabilization Hardware	Implanted PEEK headpost + clamp	CT-based individualized 3D-printed helmet + chin plate
Functional Voxel Size	0.5 mm isotropic	0.8 mm isotropic
Slice Orientation (Functional)	Axial	Coronal
Echo Time (TE)	15 ms	17 ms
Parallel Imaging Factor	GRAPPA = 2	GRAPPA = 3
Structural Sequence	2D T2-weighted imaging	3D T2-weighted SPACE
Structural Resolution	0.133 × 0.133 × 0.5 mm ³	0.2 mm isotropic
CT Usage	Not required	Required for individualized helmet design
Respiratory Monitoring	Optional respiratory pillow + LabChart	Primarily camera-based monitoring

Restraint Architecture

Body restraint + coil
housing system

MRI chair + individualized
helmet system

1.3 Harmonization Statement

The Western and McGill MRI protocols are conceptually aligned and harmonized at the level of experimental design, animal training, behavioral progression criteria, distortion correction strategy, shimming approach, monitoring standards, and safety procedures. Core functional imaging parameters, including TR and flip angle, are matched across sites to facilitate cross-site comparability.

Observed differences reflect hardware constraints, including field strength, scanner vendor, coil architecture, and head stabilization systems, rather than divergence in methodological philosophy. Spatial resolution and minor sequence parameter variations are optimized for each platform while maintaining consistency in BOLD acquisition principles.

Together, these shared design elements ensure that data acquired at both sites are scientifically comparable, while allowing each center to leverage site-specific technical infrastructure.

Comparison of the protocols was done by Sriram Jayabal, the Research Program Director-TRIDENT.

Marmoset Standard Operating Procedures

Title: Marmoset Brain Clearing Protocol

SOP No.: TRIDENT_RP_marmBC-V1

Date: 24 March 2026

Last updated: 9 Jan 2026

Created for TRIDENT, by Jesleen Saini (Western Site, Everling Lab), and compiled by Dr. Sriram Jayabal (Research Program Director, TRIDENT).

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1. Introduction

This Standard Operating Procedure, SOP, describes the workflow for whole-brain clearing, delipidation, electrophoretic immunolabeling, and refractive index matching of perfused marmoset brain tissue using SHIELD tissue preservation chemistry and the SmartBatch+ electrophoretic clearing and staining system.

The purpose of this protocol is to enable three-dimensional, high-resolution imaging of intact marmoset hemispheres or whole brains while preserving structural integrity, endogenous fluorescence, and antigenicity.

This workflow includes:

- Chemical stabilization using SHIELD epoxy chemistry
- Passive and active electrophoretic delipidation
- Electrophoretic primary and secondary antibody labeling using Radiant chemistry
- Post-label fixation
- Optical clearing via refractive index matching with EasyIndex

This protocol is optimized for intact hemispheres and whole brains and may require timing adjustments depending on tissue size.

2. Materials and Reagents

2.1 Biological Samples

- Perfused marmoset brain fixed in 4% PFA

2.2 Fixation and Storage Reagents

- 4% Paraformaldehyde in 1X PBS
- 1X Phosphate Buffered Saline, PBS
- PBSN, PBS with sodium azide

- Distilled water, diH₂O

2.3 SHIELD Chemistry

- SHIELD Epoxy
- SHIELD Buffer
- SHIELD ON Solution

SHIELD OFF Solution composition:

- 50% SHIELD Epoxy
- 25% SHIELD Buffer
- 25% diH₂O

2.4 Delipidation Reagents

- SmartBatch+ Delipidation Buffer
- SmartBatch+ Conduction Buffer

2.5 Immunolabeling Reagents

Blocking:

- Antibody Blocking Buffer
- Normal Donkey Serum, 5% final concentration
- Normal Goat Serum, 5% final concentration if using goat secondaries

Primary Labeling:

- SmartBatch+ Radiant Buffer
- Primary antibodies
- Fluorescent dyes

Secondary Labeling:

- SmartBatch+ Secondary Device Buffer
- Secondary Sample Buffer
- Secondary antibodies
- Fluorescent dyes

2.6 Optical Clearing and Index Matching

- EasyIndex
- Refractive index matched immersion oil

3. Equipment

- SmartBatch+ Clearing and Labeling System
- Radiant pH Catalyst and Radiant Column
- Clearing Cups and Batch Staining Cups
- Orbital shaker
- 37°C and 45°C incubators
- 4°C cold storage
- Light sheet microscope
- Certified chemical fume hood

4. Procedures

STEP 1 PERFUSION & FIXATION

1. Marmosets are perfused with 4% PFA, following perfusion
2. Store in PFA for 1 week
3. Following storage PBSN for 1 week
4. Extract brain and store in PBSN at 4°C

STEP 2 SHIELD OFF

Samples can now be stored in PBSN at 4°C for up to 2 months. Or proceed with epoxy.

5. Incubate samples in SHIELD OFF Solution for 6 days at 4°C with light shaking. Refresh the solution on day 3.
 - SHIELD epoxy 20 mL (50% v/v)
 - SHIELD buffer 10 mL (25% v/v)
 - diH₂O 10 mL (25% v/v)

STEP 3 SHIELD ON

6. Incubate samples in 40 mL SHIELD ON for 24 h at 37°C with light shaking.

STEP 4 BEGIN DELIPIDATION

Samples can now be stored in PBSN at 4°C for up to a year without significant loss of fluorescence signal and structural integrity. Or proceed with delipidation.

7. Incubate samples in Delipidation Buffer for 24 h at 45°C with light shaking.
This step can be extended if needed. Do not skip.

STEP 5 DELIPIDATION IN SMARTBATCH+

This process can take 35 days or more for intact-hemisphere AND 56 days or more for whole brain, depending on the size of the brain. Delipidation buffer requires refreshing every 3 days and Conduction buffer needs refreshed every 10 days.

8. Turn off Electrophoresis Power and Auxiliary Power (if not already).
9. Open the lids and remove the Clearing Cup from the Chamber.
10. Wash SmartBatch+ thoroughly with diH₂O.
11. Pour a whole bottle of Conduction Buffer into the Chamber (450mL).
12. Leak test a Clearing Cup and fill it with ~50 mL Delipidation Buffer.
13. Load the sample into the cup and place the cup in the chamber.
14. Turn on the Auxiliary Power. Close the Chamber Lid and the Case Lid. Press "Preset" until it indicates Clearing Mode. Set timer for 72 hours. Turn on the Electrophoresis Power.

Each time the Delipidation buffer is replaced, complete steps 7, 8, 11-13. Each time Conduction buffer is replaced, complete steps 7-10 and 12-13 or steps 7-13 if both buffers are replaced at the same time.

STEP 6 COMPLETE DELIPIDATION

15. Turn off Electrophoresis Power and Auxiliary Power.
16. Open the lids and remove the Clearing Cup from the Chamber.
17. Remove samples and place in PBSN at 4°C on shaker.
18. Carefully rinse the Clearing Cup with distilled water and store in its storage solution.
19. Wash the system and shut down. Save any Conduction buffer that has been used for <10 days. Record hours remaining.

STEP 7 OPTIONAL, BUT RECOMMENDED, CLEARING CHECK - INDEX MATCHING

20. Incubate in 50% EasyIndex in 50% distilled water (v/v) shaking at 37°C for 2 days in a sealed container. Protect from light.
21. Incubate in 100% EasyIndex at 37°C for 2 days or until transparent.

After index matching, the sample should be clear enough to easily see through while submerged in EasyIndex. To check using the light sheet microscope, mount on the sample holder, place in the LSM sample chamber, and leave in the RI matched oil at least overnight. Image using the autofluorescence channel (488 nm).

Wash out the EasyIndex with PBSN for 2 days at 4°C, refreshing daily, or until opaque and the solution is not oily. If the sample is not fully delipidated, return to step 4 and delipidate more. If the sample is sufficiently delipidated, continue with step 8.

STEP 8 BLOCKING

22. Incubate sample in 40 mL Antibody Blocking buffer with 5% Donkey Serum for 5 days at 37°C with shaking.
 - a) 40 mL Antibody Blocking Buffer + 2 mL Normal Donkey Serum*If you are using Goat secondaries, use 5% Goat Serum in Antibody Blocking Solution instead.*

STEP 9 RADIANT PRIMARY LABELING

22. Pre-incubate the sample(s) in SmartBatch+ Radiant Buffer 3 days before

labeling. Cover from light and shake at RT. Refresh buffer at least once (on Day 2).

STEP 10 BEGIN RADIANT LABELING IN SMARTBATCH+

23.Refresh with more SmartBatch+ Radiant Buffer. Allow the sample to shake in the refreshed buffer for at least 1 additional hour.

Radiant experiments in SmartBatch+ run 35 hours, so plan start time accordingly.

Prepare the cup and device for primary labeling:

- a. Cup preparation: Carefully rinse, dry, and check cup for leaks.
- b. Device preparation:
 - i. Wash the device. Place the Radiant pH Catalyst into the column as far down as possible. Drop the loaded Radiant Column into the hole in the reservoir lid and place the magnetic lid back in place on top of the column.
 - ii. Add SmartBatch+ Radiant Buffer to the chamber according to the sample cup size:
 1. Batch Staining Cup: 365 mL
- c. Load the sample cup:
 - i. Fill the sample cup with the following amounts of Radiant buffer.
 1. Batch Staining Cup: 40 mL
- d. Add antibodies/ dyes to the Sample Cup: Add the appropriate amount of antibody to the cup.
- e. Add Normal Serum:
 - i. Use Normal Donkey Serum if you will use donkey host secondaries, and Normal Goat Serum for goat host secondaries.
 1. Batch Staining Cup: 2400 μ L
 - ii. Mix well.
- f. Load the samples into the cup, load the cup into the device, and start the experiment. Turn on the Auxiliary Power while the lid is open and ensure that the sample cup rotates and is mixing well.
 - i. Press "Preset" to change to Labeling 1.
 - ii. Check the settings: 30°C, 90V, and 350 mA current limit.
 - iii. Change the timer to the appropriate time based on the sample cup:
 1. Batch Staining Cup: 35 hours
 - iv. Turn on Electrophoresis and Timed Shutdown (3500).
 - v. Normal starting values for current and voltage: Current

150-190 mA, voltage: 90V

STEP 11 FINISH RADIANT LABELING AND FIX

24. Move samples to PBSN, wash overnight at RT with light shaking, refreshing the solution at least once. If your sample contains fluorescence, protect from light.
25. Carefully rinse Sample Cup with distilled water and store it in its storage solution.
26. Remove the Radiant Column from the device and remove the Radiant pH Catalyst bag from the Column and dispose of it.
27. Wash the device with diH₂O before shutting it down.

PFA fixation is recommended to prevent antibody dissociation.

If you are not ready to do a 2-day PFA fix, store samples in PBSN at 4°C short-term. Extended time before fixing can result in antibody dissociation from epitope binding sites.

28. Incubate samples in 4% PFA in 1X PBS for 2 days at RT with light shaking and protected from light.

STEP 12 SECONDARY LABELING

If you are ready to complete secondary staining immediately after PFA fixation, continue to the next step. Otherwise, move samples to PBSN and store at 4°C protected from light. *You do not need to PBSN wash between PFA and secondary sample buffer.*

29. Incubate samples for 24 hours at 37°C in Secondary Sample Buffer with light shaking, refreshing the solution once.
30. Around 4 pm, prepare the cup and device for secondary labeling.
 - a. Cup preparation: Wash and leak test the correct size sample cup.
 - b. Device Preparation: Wash and dry the device. Add 300 mL of SmartBatch+ Secondary Device Buffer into the Chamber.
31. Prepare samples and solutions:
 - a. Coat the membrane of a leak-tested cup with Secondary Sample buffer
 - b. Fill the sample cup with Secondary Sample Buffer:
 - i. Batch Staining Cup: 40 mL
 - c. Add secondary antibodies/ dyes to the Sample cup.
 - d. Add Normal Serum
 - i. Batch Staining Cup: 2400 µL
 - e. Load the samples into the cup. Check for and remove any bubbles.
32. Load the cup into the device and start the experiment.
 - a. Place the sample cup into the Chamber.
 - b. Turn on the Auxiliary Power and ensure the sample cup rotates.

- c. Close the Chamber Lid and Case Lid.
- d. Press the “Preset” button until the Labeling 2 preset is highlighted.
- e. Check the settings: 30°C, 90V, and 400 mA current limit.
- f. Change the timer to 18 hours.
- g. Turn on Electrophoresis and Timed Shutdown.
 - i. Normal starting values: current 400mA, voltage: 55-65V

STEP 13 FINISH SECONDARY LABELING

33. Start the post secondary electrophoretic wash:

- a. Open the device and remove the Sample Cup.
- b. Remove the samples from the cup.
- c. Discard the antibody buffer cocktail from the sample cup.
- d. Fill the cup with a small amount of Secondary Sample Buffer, swirl gently and discard. Repeat a second time.
- e. Fill the cup with Secondary Sample Buffer and return samples to the cup.
- f. Place the sample cup back into the device, lining up the hex pieces.
- g. Turn on Auxiliary Power and ensure that the sample cup rotates.
- h. Change the timer to 3:00 (3 hours).
- i. Turn on electrophoresis and start the timer.
- j. Repeat a-i. In total, this is a 6-hour wash, with a refresh halfway.

34. Move the samples to PBSN and wash until the end of the day at RT with light shaking, refreshing the solution once. If your sample contains fluorescence, protect from light.

35. Clean the device and cup.

- a. Carefully rinse the Sample Cup with distilled water and store it in its storage solution. Always keep the membrane hydrated.
- b. Wash and shut down the device.

STEP 14 SAMPLE WASHING AND FIXATION

36. At the end of the day, wash samples in PBSN overnight at RT, gently shaking, and protected from light.

Secondary antibodies will be fixed. If you are not ready to place samples in PFA for 2 days, keep samples in PBSN at 4°C short-term. Extended time before fixing can result in antibody dissociation from epitope binding sites.

37. Prepare a solution of 4% PFA in 1X PBS and incubate samples for 2 days at RT with light shaking protected from light.

38. Transfer samples to PBSN. Wash for at least 6 hours, refreshing the PBSN halfway through.

DAY 20 INDEX MATCHING

If a sample will not be imaged until a later date, store the sample in PBSN.

39. Incubate in 50% EasyIndex in 50% distilled water (v/v) shaking at 37°C for 2 days in a sealed container to prevent evaporation. Protect from light.
40. Incubate in 100% EasyIndex at 37°C for the same duration or until transparent.

After index matching, the sample should be clear enough to easily see through while submerged in EasyIndex.

If a sample has been index matched and needs to be recovered and saved or reprocessed, the sample should be washed in PBS at RT with gentle shaking overnight and stored. You can store samples in EasyIndex at RT but be aware that they can take on a more yellow color over time that does not affect imaging.