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SMART brain mapping

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We use this protocol and it's working

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Abstract

This protocol is for the Serial sectioning and clearing, 3D Microscopy, semi-Automated Reconstruction and Tracing (SMART) strategy for high-throughput brain mapping. The SMART brain mapping pipeline of marmoset brains includes viral labeling (optional), preprocessing (post-fixation), serial sectioning, tissue clearing and staining (optional), VISoR2 volumetric microscopy, semi-automated whole-brain reconstruction, and fiber tracing (optional).

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Shenzhen Institutes of Advanced Technology (SIAT), Chinese Academy of Sciences (CAS), and conducted in an AAALAC-certified facility. Researchers applying this protocol should ensure compliance with local ethical and regulatory standards for animal experimentation.

Materials

Animal

Marmosets were individually housed under conditions that adhered to institutional guidelines of CEBSIT, with a 12-hour light/dark cycle (light on from 7 a.m. to 7 p.m.), a humidity and temperature-controlled environment (27°C to 30°C), and ad libitum access to food and water. A 7.5-year-old male marmoset was used in this protocol.

▲ **CAUTION:** The animal studies and procedures were approved (ION-2019011) by the Animal Care and Use Committee of the Center for Excellence in Brain Science & Intelligence Technology (CEBSIT), Chinese Academy of Sciences, Shanghai, China.

Reagents

Reagents for surgery and viral labeling

l Anesthetics

for marmoset: Isoflurane (1.5% final concentration, delivered in 100% oxygen, RWD, cat. no. R510-22-10) may be used.

▲ **CAUTION** Isoflurane is a highly volatile substance and a proper device like anesthesia induction box or breathing mask for animal should be used

l AAV-Cap-B10, [ssAAV.hSyn.EGFP.WPREs.SV40pA]

Reagents for animal perfusion and tissue fixation

l All reagents can be purchased from common vendors.

l Gibco 10× phosphate-buffered saline (PBS), pH 7.4 (Fisher Scientific, cat.no. 70011044)

l Paraformaldehyde (PFA), 8% (Solarbio Life Science, cat.no.P1112)

▲ **CAUTION** PFA is toxic. Perform all procedures in a fume hood. Avoid inhalation and exposure to the skin or the eyes.

▲ **CRITICAL** Dilute 8%PFA to 4% final concentration to prepare HMS solution described in “Reagent setup”.

l Acrylamide (Aladdin, cat.no.A108465)

l N,N′-Methylenebisacrylamide (Sigma, cat.no. M7279)

l 2,2'-Azobis
[2-(2-imidazolin-2-yl) propane] dihydrochloride (VA-044), (Wako, cat.no.
223-02112)
▲ **CRITICAL** VA-044 is a
heat -sensitive azo polymerization initiator, thus the powder or the aqueous
mixture should be stored at 4°C until polymerization step.

Reagents for
tissue embedding
l Bovine serum
albumin (Sigma, cat.no. V900933)
l Hydrogel
monomer solution (4%)
▲ **CRITICAL**
4% HMS can be prepared as described in 'Reagent setup'.

Reagents for
smart brain mapping
l Triton X-100 (Sigma, cat. no. T928)
l Gibco 10×
phosphate-buffered saline (PBS), pH 7.4 (Fisher Scientific, cat.no. 70011044)
l PC-300
(Solarbio, cat.no. P6840)
▲ **CRITICAL** PC300 is
used here as a preservative.
l Agarose, low
melting (Sangon, cat.no. A600015-0025)
l 4',
6-diamidino-2-phenylindole (DAPI, Beyotime
Biotechnology, cat. no. C1006)
l NeuroTrace™
640/660 deep red fluorescent Nissl stain (NT640, ThermoFisher, cat. no. N21483)
l Iohexol (
Hisyn Pharmaceutical, cat. no. 29242990.99)
l Urea (
Sangon, cat. no. A600148-0002)
l 2,2′,2″,-nitrilotriethanol
(Sigma, cat.no. V900257)
l Sodium
chloride (Sigma, cat. no. S5886)
l Potassium
chloride (Macklin, cat. no. C16403322)

l Sodium
phosphate dibasic (Sigma, cat.no. S5136)
l Sodium
bicarbonate (Sigma, cat. no. S5761)

Equipment
l 50 ml conical tube (Corning, cat. no. 352070 or Greiner, cat. no. 227261)
l 24-gaugue catheter (Introcan-w,
cat.no. 4254074B)
l 2ml Sterilized Microcentrifuge Tubes, enzyme-free(Keewin, cat.no.
A211-CT02002)
l Inhalation Anesthesia Machine (RWD, cat.no.R530)
l Oscillating Incubator(Shanghai Zhichu Instrument, cat.no.ZQTY-90E)
l Oscillating Incubator(Shanghai Zhichu Instrument, cat.no.ZQZY-A8)
l BOD Cooling Incubators（Shanghai Zhicheng, cat.no.ZXMP-R1230）
l Circular Decolorization Shaker(Dlab, cat.no.SK-0180-S)
l Vortex mixer(Ohaus, cat.no.VXMNAL)
l Ice maker(Xueke, cat.no. IMS-60)
l Tissue culture plate, 6 well(Falcon, cat.no. 353046)
l Screw-thread Wide-mouth Bottle, 125ml(CNM, cat.no.SGEQ-2180125)
l Vacuum desiccator(Shanghai Yueci, cat.no. PC-3)
l Vibroslicer (Precisionary Instruments, cat.no.Compresstome VF-800)
l VISoR2 imaging systems (Bitelligen, cat.no.VISoR M1)
l 9.4T/12-cm MRI(Bruker Biospin)
l Reagent bottle 1000ml
l Chinese brush
l Mounting glass
l Coverlip
l Patching chamber
l Imaging chamber
l Digital Display Refractometer(ATAGO, cat.no.PAL-RI)
l Microwave
oven
l Fume hood
l Heating blanket

Data analyze tools
l VISoR Reconstruction
(https://github.com/SMART-pipeline/Volume-reconstruction)
l

I CABLE (https://github.com/BrainCABLE)

Reagent setup

1×PBS, 20L stock solution In ddH_2O add 160gNaCl, 4g KCl, 28.4g Na_2HPO4, 5.4g KH_2PO4. Stir until dissolved and the solution is stable at room temperature for 2 months.

40% (w/v) acrylamide solution Prepare 16g of acrylamide and then add distilled water to 40ml. Shake until fully dissolved and then store at 4°C for up to 1 month.

2% (w/v) bisacrylamide solution Prepare 0.8g of N,N′-Methylenebisacrylamide and then add distilled water to 40ml. Shake until fully dissolved and then store at 4°C for up to 1 month.

4% HMS 40ml 4% Hydrogel monomer solution (HMS, 4%) which was prepared for perfusion by mixing 4ml 40% acrylamide (4% final concentration), 1ml 2% bisacrylamide (0.05% final concentration), 4ml 10×phosphate buffered saline (PBS) (1×final concentration), 20ml 8% (w/v) paraformaldehyde (4% final concentration), 11ml distilled water and 0.5g VA-044 thermal initiator (1.25% final concentration). The preparation process should be carried out on ice and in fume hood. The final mixture can be stored at 4°C for 1-2 week.

▲ **CRITICAL:** the mixture could polymerize as hydrogel when the temperature rises.

20%BSA Dilute 8g of Bovine serum albumin powder in distilled water to final volume 40ml.

▲ **CRITICAL:** the solution will generate foam after shaking. It is necessary to wait until the foam completely dissipates before conducting the volume-fixing. The solution should be prepared before the day of experiment and keep in 4°C for no more than 1 week.

4%Agrose Dilute 1.6g Agarose powder in ddH_2O to 40ml. The agarose would melt through heating in a microwave oven.

5% TritonX-100 in PBS (PBST) Dilute 5ml Triton X-100, 10ml 10×phosphate buffered saline and 0.1ml PC-300 into 84.9ml distilled water. The solution keep stable for 1 month.

Preparation of slice

staining solution On the day of experiment, mix 40ml fluorescent dyeing solution containing 35.36 ml 4′,6-diamidino-2-phenylindole (DAPI) stock

solution (79.4% final concentration) for nucleus , 0.4ml NeuroTrace 640/660 deep-red fluorescent Nissl stain (NT640, 1% final concentration), 0.2ml Triton X-100 (0.5% final concentration), 0.04ml PC-300(0.1% final concentration), 4ml 10×PBS（1×final concentration)

▲ **CRITICAL:** the final concerntration of NeuroTrace 640/660 deep-red fluorescent Nissl stain should vary depend on the sample relating to the slice thicknenss and tissue density.

PuClear refractive index matching solution The RI matching solution is prepared by mixing 650g Iohexol (50 wt%) , 300g urea (23wt%), 140g 2,2′,2″,-nitrilotriethanol (11wt%) and 210ml distilled water(16wt%). Then place the reagent bottol into Oscillating Incubator(Shanghai Zhichu Instrument, cat.no.ZQZY-A8) shaking for 48h at 180rpm in 37°C. Test the final RI to 1.52 using Digital Display Refractometer(ATAGO, cat.no.PAL-RI). The matching solution keep stable in 1 month.

Whole-brain Viral labeling (Optional)

1 **For Marmoset**

Note

CAUTION: An appropriate viral labeling method could be altered according to certain experimental design.

1.1 Implement fasting and water restriction for at least 6 h before surgery.

Note

Caution: Fasting and water restriction are required to prevent gastric regurgitation.

1.2 Anaesthetize the marmoset with 1.5% -3% isoflurane inhalation in oxygen before surgery.

Note

Critical: The anesthetic concentration should be adjusted per the animal's condition intraoperatively.

1.3 Shave the skin over the tail vein and sanitize with an ethanol scrub.

1.4 Inject of 2 mL of the tracer (AAV-Cap-B10, [ssAAV.hSyn.EGFP.WPREs.SV40pA], 1×10^{13} gc/mL) through a 24-gauge catheter by tail vein injection over several minutes.

1.5 Recover the animal on a warm blanket (37 °C) until they regained normal motor functions.

1.6 Set the animal back to cages and monitor closely for normal behavior over the 4d, followed by daily
obervations thereafter.

1.7 Euthanize the marmoset for brain harvest 8 weeks after injection.

2 **For Mouse**

2.1 Anaesthetize the mice with 1.5%-2.0% isoflurane inhalation in oxygen before surgery.

2.2 Inject 100μl of the same crossing BBB viral labeling tracer as used on marmoset(AAV-Cap-B10, [ssAAV.hSyn.EGFP.WPREs.SV40pA], 1×10^{13} gc/mL, 1:10000 dilution) through 24-gauge catheter by retro-orbital vein injection over several minutes.

Note

CAUTION: If bleeding occurs after injection, conduct hemostasis and anti-inflammation treatment in time and make a record. The injection volume may be affected by bleeding.

2.3 Recover the animal on a warm blanket (37 °C) until they regained normal motor functions.

2.4 Euthanize the mice for brain harvest 8 weeks after injection.

Specific circuit labeling (Optional)

3 **For Marmoset**

3.1 Anaesthetize the marmoset with 1.5-3.0%(v/v) isoflurane inhalation in oxygen by placing the animal in an induction box before surgery.

3.2 Connect the animal to the bench mask with about 1.5% -3.0% isoflurane-oxygen gas flow, using a stereotaxic apparatus to fix the head of the marmoset. The optional step is performing oral tracheal intubation, then connecting the gas anesthesia pipeline and equipment.

3.3 Place the temperature probe into the anus or between the belly and the blanket, check the temperature, heart rate and SpO_2 during the process.

3.4 Screw the stereotaxic frame to the base plate with the anterior/posterior (A/P) rail and medial/ lateral (M/L) rail.

Note

CRITICAL: Avoid head movement during surgical procedures to reach the accurate determination of stereotaxic coordinates.

3.5 Shave the skin with washes of povidone iodine, followed by 75% ethanol.

3.6 Incise the skin over the sagittal midline with a sterile surgical blade.

Note

CRITICAL: Surgical instruments and procedures must be sterile.

3.7 Check the position of the microsyringe orthogonal to the x/y/z axes, and place the skull in a horizontal position.

3.8 Measure the injection sites according to brain atlas or MRI result; mark the borders around the craniotomy: the occipital region (ventral visual area 4) of the left hemisphere (AP, -3.79mm; ML, 6.79mm) with a sterile pen.

Note

CAUTION: When using the anatomical atlas for stereotaxic injections, actual injection coordinates may deviate due to individual biological variations in brain morphology, size, and landmark positioning.

3.9 Perform a craniotomy at the marked position with a dental drill and keep the bone fragment in sterile saline buffer.

Note

CRITICAL: If bleeding occurs after injection, conduct hemostasis and anti-inflammation treatment in time. Avoid puncturing the dura mater during this procedure.
Make sure the injection needle is not blocked if it touches blood.

3.10 Gently rinse the drilled area with sterile saline buffer, and employ the microsyringe injector which is filled with 1000 nL rAAV mixture (AAV2/9, [rAAV-hSyn-SV40-NLS-Cre] 1/10000, [rAAV-CAG-DIO-tdTomato] 1/4) to administer the virus gradually into the depth within the target brain region mentioned in step 3.8 (AP, -3.79mm; ML, 6.79mm; DV, 9.75mm). The injection is performed at rate of 200 nL/min and maintain above 20 minutes (including 5 min both following insertion and before withdrawal of the microsyringe).

Note

CRITICAL: Make sure the injection needle is not blocked if it touches blood.

3.11 Place the bone fragment back in its original position and apply some bone wax around the borders.

3.12 Suture the incision. Administer the antibiotics to the animal for 3 days after surgery.

3.13 Return the animal to the home cage after it is fully alert. Monitor the animal for a further 1–2 h in the home cage and twice a day until total recovery

4 **For Macaque**

4.1 For macaques, the stereotaxic injection process could refer to the above method for marmosets.

Note

CRITICAL: Injection sites could be further confirmed from reconstructed whole-brain images

Brain brain acquisition

5 **For Marmoset**

5.1 Transcardial perfuse the marmoset after aneasthetized with 1.5% isoflurane.

5.2 Infuse 500mL PBS at speed 30mL/min in 16min, and 100mL 4% HMS at rate of 40mL/min in 2.5min, 250mL 4%HMS (30mL/min) in 8min, 1L 4% HMS(20mL/min) 50min.

Note

CRITICAL: All the solution should be stored and operated in 4℃.

5.3 Extract the brain immediately after perfusion, within 30 min.

6 **For Macaque**

6.1 Anesthetized macaques by 10 mg/kg ketamine hydrochloride injection (i.m.,50 mg/mL) and maintained with 20 mg/kg pentobarbital sodium (i.m.,40 mg/mL)

6.2 Perform transcardial perfusions thirty minutes after anesthesia, by sequentially perfusing with the following solutions at the specified speed: PBS 8 L (37°C, 10 mL/s), PBS 1 L (4°C, 1.5 mL/s), 4% HMS 1L (4°C, 1.5 mL/s), and 4% HMS 1 L (4°C, 0.3 mL/s)

6.3 Extract the brain immediately after perfusion

7 **Mouse(Including vasculature labeling)**

7.1 After anesthetizing the mice via inhalation with 1.5% isoflurane in oxygen, inject 75 µL of DyLight 488- or DyLight 649-conjugated *Lycopersicon esculentum* lectin via retro-orbital route.

Note

CAUTION: This is a optional step according to the experimental requirements. The labeling reagent can be replaced as per the experimental.

7.2 One hour post-injection, administer secondary anesthesia via intraperitoneal injection of a ketamine/xylazine mixture (dose:10 mg/kg ketamine hydrochloride; 1 mg/kg xylazine hydrochloride).

7.3 Perfuse the follow solutions in sequence: 30 mL of PBS (37 °C, 10ml/ min), 30 mL of PBS (4 °C , 10ml/ min), 30 mL of 4% PFA (4°C, 10ml/ min), and 75 µL of lectin dissolved in 30 mL of ice-cold PBS (4°C, 3ml/ min).

Note

CRITICAL: Remove the lectin solution when referring to this procedure for perfusion without the requirement of labeling vasculature

7.4 Extract the brain immediately after perfusion and store temporarily in 4% PFA.

Post-fixation and embedding

8 **For standard samples**

8.1 Immerse the brain sample in 4%HMS and store at 4 °C for 2–7 days, depending on brain size and tissue density (typically 2 days for mouse brains; 7 days for primate brains)

8.2 Transfer the brain sample to the embedding solution, a 1:1 mixture of 4% HMS (2% final concentration) and 20% bovine serum albumin (10% final concentration) and incubate in the sulution for for 0.5–7 days in 4°C, again depending on tissue size and density

8.3 Transfer the brain and embedding solution to Constant Temperature & Humidity Incubator, polymerize at 37°C for 4-5 h.

8.4 Wash 3 times in PBS for 1 h each time to remove residual reagents.

9 **For immunolabeling**

Note

CRITICAL: These alterations have the potential to boost the efficiency of antibody penetration.

9.1 Post-fix the brain for 1-7days in 4% PFA instead of 4%HMS.

Note

CRITICAL: The time of post-fix is typically 1 day for mouse brains, 4 days for monkey brains and 7days or longer for human brains.

9.2 Wash the brains with PBS three times over a 24-hour period at RT to remove the PFA.

9.3 Immerse the brains in 10% fish skin gelatin solution(37°C，liquid) for 30minutes (the larger brain could extend the immersion time to 4 hours)

Note

CAUTION: The gelatin solution is in a solid state at room temperature. When using it, preheat it to 37°C in advance to restore it to a liquid state.

9.4 Locate the brain and 10% fishskin gelatin solution to a mold box of appropriate size and wait for the gelatin to set.

9.5 Place the mold containing the brain at 4°C for more than 4 hours.

9.6 Demold, trim the embedded material to a suitable size and make corner cuts for identifying the orientation.

9.7 Immerse the embedded brain in 4% PFA to solidify the gelatin for more than 20 hours.

Note

CRITICAL: Gelatin fixed by PFA can remain in a solid state at 37°C or higher temperatures.

Sectioning and clearing

10 **Serial sectioning**

10.1 Trim the embedding block and and mark a notch on the left ventral side to preserve orientation for whole-brain reconstruction.

10.2 Stick it onto the stage of vibroslicer with glue, fill the gap space with melting 4% agarose and wait for the agrost solidify.

Note

CRITICAL: Select a suitable vibratome according to the size of the samples.

10.3 Section the embedded brain into 300-μm-thick slices from the anterior segment of the coronal plane.

Note

CRITICAL: The thickness of sections can be altered (normally reduced to 150μm or 100μm) to achieve better staining effects according to the molecular sizes of dyes or antibodies.

10.4 Utilize a soft Chinese brush to gently lift out the brain slices. Mark the brain slices in a squential counting.

10.5 Place the brain slice into three 6-well cell culture plates, 3-5 with known serial numbers are placed in one well filled with PBS

Note

CRITICAL: For brain samples: use 12-well plates for mouse brains, 6-well plates for marmoset brains, and individual petri dishes for monkey and human brains.
Brain slices should be arranged cyclically according to the count per cycle, with clear distinguishability of slices within the same well.

Expected result

- Marmoset brains would be yielding approximately 70-105 slices per brain.
- Whole macaque brains generate approximately 200–250 slices per brain.
- Mouse brains yield approximately 40–50 slices per sample.

10.6 Store the plates at 4°C

11 **Tissue Clearing**

11.1 Treat the brain slices with a high concentration of Triton X-100 solution (5% in PBS) for1-4 days at 37°C in Oscillating Incubator at rate of 60rpm.

Note

CRITICAL: The clearing time for mouse brains is 1 day, while that for monkey and human brains is 4 days.

Staining

12 **For general staining**

12.1 Stain the brain slice with slice staing solutin for DNA and nissl stain for 2-4 days.

Note

CRITICAL: The staining time for mouse brains is 2 days, while that for monkey and human brains is 4 days.

12.2 Wash 4 times with PBS after stain.

13 **For immunolabeling**

13.1 Block the brain slices in 0.5M Glycine in 0.3%PBST for 4 hours at 37°C.

Note

CRITICAL: The BSA or serum used in conventional blocking may quench Nissl fluorescent dyes, glycine is employed here to block the nonspecific effects of aldehyde groups.

13.2 Wash 1 hour in PBS at RT.

13.3 Incubate the brain slices in primary antibodies dissolved in 0.3%PBST for 3-7days at 4°C.

Note

CRITICAL: The incubating time for mouse brains is 3 day, while that for monkey and human brains is 4-7 days.

Note

CRITICAL: When co-staining with different antibodies, pay attention to distinguishing the host species.

13.4 Wash 4 times with 0.3%PBST, 1-2 hours each time.

13.5 Perform the general staining if needed.

Note

CAUTION: General staining can be performed simultaneously with the secondary antibody, ensuring accurate final concentrations of all dyes.

Mounting and RI matching

14 **Mounting**

14.1 Trim off the excessive embedding, and remain the notch of slices.

14.2 Immerse the brain slice into 4%HMS, and transfer the slices on the hydrophilic mounting glass with the Chinese brush setting the left ventral side of each brain slice in one direction.

14.3 Stick suitable number in order and clip the mounting glass in the patching chamber

14.4 Drop an excessive amount of vacuum-treated HMS onto the brain slices, overlay a hydrophobic cover glass on the top

14.5 Fix the chamber and place it into a sealed box with few ddH_2O below the chamber.

14.6 Put the box to Constant Temperature & Humidity Incubators at 37°C for 3-4h.

14.7 Remove the cover glass and place the mounting glass with brain slices into PBS.

15 **Refractive index matching**

15.1 Fix the mounting glass into imaging chamber and fill the PuClear RI matching solution.

15.2 Incubate in the brain slices overnight to facilitate optical transparency.

VISoR2 Microscopy

16 10X/NA0.3 objectives equipped in these systems are used to generate raw images of the slices at 1×1×2.5 μm^3 pixel resolution.

17 Triple-channel imaging of a marmoset brain generates 20 terabytes (TB) images after compression, with an approximate compression ratio of 8:1.
Dual-channel imaging of a rhesus macaque brain generates 500 TB raw images.
Four-channel imaging of a mouse brain generates 1 TB raw images.

Whole-brain reconstruction

18 Volume stitching of VISoR2 raw images are performed with custom codes (https://github.com/SMART-pipeline/Volume-reconstruction).

Semi-automated tracing (Optional)

19 The single axon reconstruction of the marmoset brain (CJ004) is semi-automatedly performed with the NeuroFly (**beanli161514/neurofly: Some tools for neuronal image analysis**) software.

19.1 Install Neurofly following the repository in Github: **beanli161514/neurofly: Some tools for neuronal image analysis**

20 Use NeuroFly.
Whole-brain Single Neuron Reconstruction Using NeuroFly

21

Protocol references

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