Transcardial Perfusion

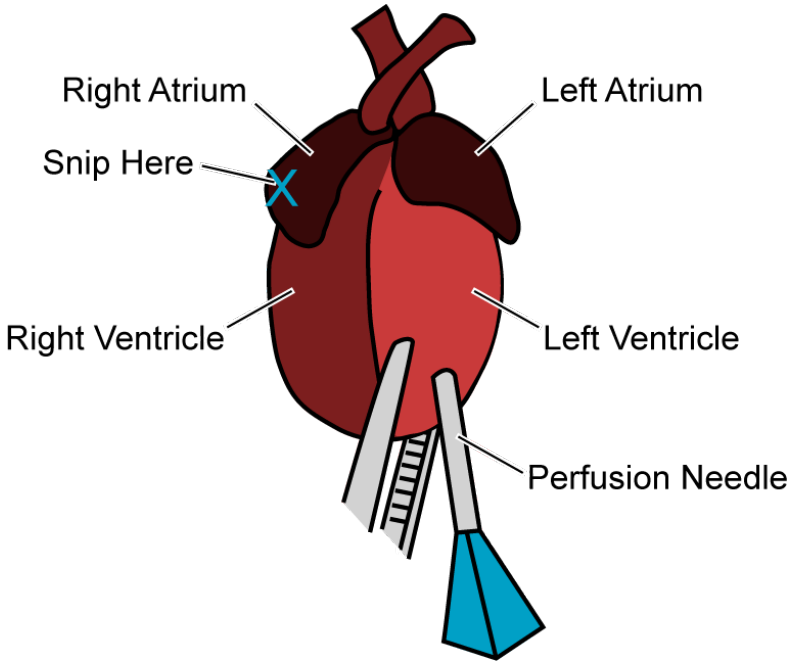
MH_03.01.2019

Materials Needed:

Aluminum-wrapped Styrofoam
Plastic lid (to capture runoff)
Labmat (on bench and on styrofoam)
30 mL syringe with butterfly needle with cut tip
*Fill with 30 mL PBS and push PBS through tubing so all air bubbles are expelled.
Perfusion pump: 100-120 mL/hour
Dissection tools
1 tube with 2 small drops heparin for blood
1 tube for tail
2 conicals with ethanol or formalin fixative
30 mL/mouse 0.1M PBS + heparin (1:100)
70% ethanol bottle

1. Make mixture of ketamine:xylazine:acepromazine (4:2:1) sufficient for anesthesia of all mice (~30 µL/ 20g mouse). Record ketamine used in controlled substance log book. Draw all anesthesia into one syringe.
2. Apply anesthesia to one mouse via intraperitoneal injection, and place mouse in bucket long enough for anesthesia to take effect. Apply a hard toe pinch until mouse no longer reacts, ensuring that the mouse can no longer feel pain before proceeding.
3. Place mouse, abdomen-up, on Styrofoam block. Spray mouse abdomen with 70% ethanol. Grasp skin below ribcage with forceps and cut skin with scissors from middle up either side towards the armpits, cutting through ribcage.
   * Avoid blood vessels and organs. Diaphragm should carefully be cut circumferentially.
4. Remove pericardium and peripheral fat to expose heart. Hold back ribs with hemostats.
   * At this point blood can be taken via syringe from the right ventricle if desired. 200-300 µL should be sufficient.
5. Place blunted butterfly needle into left ventricle and cut right atrium. Push PBS through syringe by hand or using perfusion pump. Liver should lose pigment and effluent should be dark blood then become more PBS as perfusion continues.
6. After perfusion is complete, remove brain, spinal cord and other desired parts and transfer to fixative.
   * Brain can be removed by carefully drilling hole with scissors at olfactory bulb junction and spreading scissors apart so skull splits in half.

Perfusion Schematic



Perfusion Solutions:

<u>4% PFA in 0.1 M NaPO4</u> (1L)

Dissolve into 500ml diH20:
- 11.36 g sodium phosphate dibasic
- 2.76 g sodium phosphate monobasic

Once dissolved:
- Add 40g PFA (powder)
- Fill to 1L with diH20

- Weigh PFA under the hood and using bench pads
- Heat and stir (in chemical hood).
- The PFA will dissolve around 60°C (use the thermometer). Do not let the temperature exceed 65°C (remove from hot plate temporarily if necessary)
- Once dissolved but still foggy, pH to 7.4 with NaOH (it should make the PFA clear)
- Prepare a big filter funnel using Whatman filter paper
- filter the 4% PFA into a liter bottle/w lid
- Store the PFA covered at 4°C

Immunohistochemisty/Immunofluorescence

MH_08.02.2018

Day 1

1. Label slides with antibody and treatment to be used.
2. De-paraffinize slides 2X 5 min in fresh xylenes, then in a descending ethanol series (1 min each): 100% 100% 95% 80% 70%
3. **Formic Acid Retrieval** (If necessary, do here.)
   Immerse slides in ddH_2O for 1 min, place in recycled FA for 5 min and wash in running tap H_2O for 10 min.
4. **Microwave antigen retrieval** (CA; If necessary do here.)
   a. Dilute antigen unmasking solution (Vector Labs, citric acid) 1:100 in dH_2O (2.5 mL/250 mL dH_2O/boat).
   b. Place in microwave for 15 min at 95°C.
   c. Cool for 20 min at RT.
   d. Wash slides for 10 min in running tap H_2O.
5. Immerse in freshly prepared Methanol/H_2O_2 (150 ml Methanol + 30 ml stock 30% H_2O_2) 30 min. **DO NOT GET ON SKIN OR LEAVE SPILL ON BENCH.** Then, wash in running tap H_2O for 10 min.
   *This step is not necessary for immunofluorescence.
   *May use DI water/H_2O_2 (150 ml DI water + 50 ml stock 30% H_2O_2).
6. Wash in 0.1M Tris buffer, pH 7.6 5 min. *Discard all Tris washes.*
7. Block in 0.1M Tris/2% FBS (Tris/FBS) 5 min+. *Keep blocking solution for up to 2 weeks @ 4°C.*
8. Dilute primary antibodies in Tris/FBS), and prepare humidified chamber(s) by soaking towel in the middle of the slide chamber(s).
9. Wipe excess fluid off back of slides and from around tissue and apply 200 µL of primary antibody to slides (hydrophobic pen may be used at this point if desired, but CANNOT be used for immunofluorescence). Make sure antibodies cover all sections.
10. Incubate at 4°C in humidified chamber overnight.

Day 2

1. Rinse off antibody from tissue using Tris (carefully direct spray from wash bottle around tissue, NOT directly on it).
2. Wash in Tris 5 min.
3. Block in Tris/FBS 5 min.
4. Dilute Vector biotinylated IgG 1:1000 in Tris/FBS and apply 200 µL to wiped slides.
   *For immunofluorescence, dilute fluorescent secondary antibodies 1:500 in Tris/FBS and apply 200 µL to wiped slides. *Keep slides in the dark from here on.* Incubate at 4°C in humidified chamber overnight or at RT for 3 hours. Proceed to Day 3.
5. Incubate at room temperature in humidified chamber 1 hour.
6. Rinse biotinylated IgG using Tris.
7. Wash in Tris 5 min.
8. Block in Tris/FBS 5 min.

9. Mix AB solution (Vector peroxidase standard) in Tris/FBS to a dilution of 1:000 (ie add 1 µl of A and 1 µl of B to 1ml of 0.1M Tris/2%FBS). Vortex and let sit 15 minutes before use. Then, apply 200 µL of AB solution to wiped slides.
10. Incubate at room temperature in humidified chamber for 1 hour.
11. Rinse off AB using Tris.
12. Immerse in Tris 5 min.
13. Make Vector DAB solution (1 drop of DAB per mL of Stable DAB Buffer) .
14. Apply 200 µL of DAB to each slide and incubate until a visible brown signal is seen and well developed. Development time may differ by antibody, but all sections treated with the same antibody should be developed for the same amount of time.
15. Rinse with Tris and place in dH_2O. Wash 5 minutes in dH_2O. Filter Harris hematoxylin.
16. Counterstain briefly with Harris hematoxylin (~15s, depending on age).
17. Wash in running tap H_2O 5 min.
18. Dehydrate and clear in ascending ethanol and xylenes (1 min each EtOH and 5 min each xylene)
    70% 80% 95% 100% 100% Xylenes Xylenes
19. Coverslip with cytoseal.
20. Dry in tissue processor closet overnight.

Day 3 (Immunofluorescence)

1. Filter Sudan Black. This process can take a long time, so start early.
2. Rinse off AB using Tris.
3. Wash in running tap H_2O for 5 minutes.
4. Wash in Tris for 5 min in green boats.
5. **Sudan Black Treatment** (0.3% Sudan Black B in 70% Ethanol)
   Use a control slide (usually 1 positive primary and 1 secondary only) to titrate for background reduction without changing signal intensity (usually 10 sec- 1 min). Image before and after Sudan black treatment for various times. Treat all slides identically.
6. Wash in 0.1M Tris 5 min in green boats.
7. Coverslip using non-photobleaching reagent (FluorMount with DAPI). Allow to dry completely before imaging on scanner.

Solutions and Reagents

0.5 M Tris (8 L)

Needed (mL)	Stock Solution	Final Concentration
5 L	dH_2O	
485 g	Tris base	0.5 M
240 mL	Concentrated HCl	
pH to 7.6		
To 8L	dH_2O	

Reagents

Vendor	Catalog #	Qty	Unit Price	Description
Vector Laboratories	H-3300	1	132.60	Antigen Unmasking Solution, Citric Acid Based
Vector Laboratories	H-4000	1	120.00	ImmEdge Hydrophobic Barrier Pen

Vector Laboratories	PK-6100	1	248.63	VECTASTAIN Elite ABC Kit (Standard)
Vector Laboratories	SK-4105	1	138.13	ImmPACT DAB Peroxidase (HRP) Substrate
Vector Laboratories	BA-2000	1	55.00	Biotinylated Horse Anti-Mouse IgG Antibody
Vector Laboratories	BA-1100	1	140.00	Biotinylated Horse Anti-Rabbit IgG Antibody
Sigma	199664-25G	1	66.60	Sudan Black B
Thermo Fisher	6765001	1	46.41	Shandon Harris Hematoxylin (non acidic)
Fisher Scientific	23-244-256	1	22.96	Cytoseal 60; 4 oz.
Southern Biotech	0100-01	1	45.14	DAPI Fluoromount-G

Dual *In Situ* Hybridization/Immunofluorescence MXH_04.28.2021

Adapted from ACD Standard Protocol/Cheadle/Otero-Garcia Protocols

**ACD protocol notes that tissues should be fixed in 10% NBF for 16-32 hours, and embedded in paraffin. Then, sectioned and dried overnight at RT. They suggest sectioned tissue be used in less than a year (4°C) or less than 3 months at room temperature.*

Preparing Tissue (Day 1)

Prepare Tissue

1. Bake slides in a dry oven for **1 hour** at **60°C**. Use slides within a week.
2. De-paraffinize slides 2X **5 min** in fresh xylenes, then 2x **2 min** in 100% ethanol.
3. Place slides on absorbent paper and dry in the oven from **5 min** at **60°C** or until dry.

Hydrogen Peroxide Treatment

1. Place slide horizontally in an incubation tray. Add **~5-8 drops** of RNAscope Hydrogen Peroxide to cover each section. Incubate for **10 min** at **RT**.
2. Dab solution off and move to a rack in distilled water. Move up and down 5 times. Repeat with a fresh boat of distilled water.

Target Retrieval

1. Dilute Target Retrieval Regents (RNAscope) 1:10 in dH_2O (25 mL/225 mL dH_2O/boat). Mix well.
2. Place in microwave for 15 min at 95°C.
3. Transfer slides to a slide boat with 200 mL distilled water for **15 sec**.
4. Transfer the slides to 100% ethanol for **3 min**.
5. Dry the slides in a **60°C** incubator (or **RT**) for **5 min**.
6. Draw a hydrophobic barrier onto slides with ImmEdge pen. Do NOT due for fluorescent slides. Let the barrier dry for 5 min. OPTIONAL PAUSE POINT overnight at RT.

RNAscope Multiplex Fluorescent v2 Assay (Day 2)

Protease Treatment

1. Place a wet Humidifying Paper in an incubation tray and warm for **30 min** at **40°C** (TC incubator). Keep the tray in the incubator when not in use. Insert the slides into the incubation tray.
2. Add ~5 drops RNAScope Protease Plus (Protease III-Cheadle) to cover each section and place tray into the incubator at **40°C** for **30 min** (standard) (15 min-Otero-Garcia). *Prepare RNAscope assay reagents during this step.*
3. Wash slides **2x** with **200 mL+** distilled water and slight agitation.

Preparation

1. **Wash Buffer:** Warm 50x Buffer to 40°C for 10-20 min. Add 980 mL distilled water to **20 mL** of RNAscope Wash Buffer in a 1L bottle. May need 1-2 liters/run. Mix well. Can be stored for up to **one month**.
2. **Probes:** Prepare only those probes needed. If you are only using C2 and C3, dilute in probe diluent instead of C1. Warm probes for **10 min** at **40°C**, then let cool to **RT**. Add 1 volume C2 and 1 volume C3 probes to 50 volumes C1 probe in a tube (e.g. 200 µL C1 + 4 µL C2). Invert to mix. Store at **4°C** for up to **6 months**.
3. **Reagents:** Warm AMP1-3, HRP-C1-3 and HRP blocks at **RT**.
4. **(Optional) Saline Sodium Citrate:** 175.3 g NaCl + 88.2 g sodium citrate in 800 mL distilled water. Adjust to pH 7.0 with 1M HCl. Add water to a final volume of 1 L. Sterilize by autoclaving and store at RT for up to 2 months.

Hybridize Probes

1. Remove liquid from slides. Add **4-6 drops (6 drops=180 µL)** of the probe mix to slides. Incubate in incubator for **2 hours** at **40°C.**
2. Wash slides with Wash Buffer 2x **2 min** at **RT**.
3. OPTIONAL PAUSE POINT: Store slides in 5x SSC overnight at RT.

Hybridize AMPs

1. Remove liquid from slides. Add 4-6 drops RNAScope Multiplex FL v2 *Amp 1* to each slide. Incubate in incubator for **30 min** at **40°C.**
2. Wash slides with Wash Buffer 2x **2 min** at **RT.**
3. Repeat steps 1 and 2 for Amp 2 and Amp 3. Amp 3 only requires **15 min** at **40°C.**
4. During this incubation, dilute necessary Opal Dye fluorophores in TSA Buffer (**1:1500** standard).

Develop HRP Signals

1. Remove liquid from slides. Add 4-6 drops RNAScope Multiplex FL v2 *HRP-C1* to each slide. Incubate in incubator for **15 min** at **40°C.**
2. Wash slides with Wash Buffer 2x **2 min** at **RT.**
3. Remove liquid from slides. Add 200 uL Opal 520 to each slide. Incubate in HybEZ Oven for **30 min** at **40°C.**
4. Wash slides with Wash Buffer 2x **2 min** at **RT.**
5. Remove liquid from slides. Add 4-6 drops RNAScope Multiplex FL v2 *HRP Blocker* to each slide. Incubate in incubator for **15 min** at **40°C.**
6. Wash slides with Wash Buffer 2x **2 min** at **RT.**
7. STOP HERE IF USING JUST C1 PROBE. Continue to **Immunofluorescence**.
8. Repeat steps 1-7 with *HRP-C2* and Opal 570, and again with *HRP-C3* and Opal 690.
   *Note that after additional of fluorophores, slides should be kept out of the light as much as possible.
   (4% PFA fix for **15 min** at **4°C**, then 2x **4 min** wash with PBS-Otero-Garcia)

Immunofluorescence

11. Wash in 0.1M Tris buffer, pH 7.6 **5 min**. *Discard all Tris washes.*
12. Block in 0.1M Tris/2% FBS (Tris/FBS) **30 min+**. *Keep blocking solution for up to 2 weeks @ 4°C.*
13. Dilute primary antibodies in Tris/FBS), and prepare humidified chamber(s) by soaking towel in the middle of the slide chamber(s).
14. Wipe excess fluid off back of slides and from around tissue and apply **200 µL** of primary antibody to slides.
15. Incubate at 4°C in humidified chamber **45 min** – **2 hours** at **RT** or **overnight** at **4°C**.

Day 3

16. Rinse off antibody from tissue using Tris (carefully direct spray from wash bottle around tissue, NOT directly on it).
17. Wash in Tris 5 min.
18. Block in Tris/FBS 5 min.
19. Dilute fluorophore-conjugated secondary antibody 1:500 in Tris/FBS and apply **200 µL** to wiped slides. Incubate at **RT** for **2 hours** or overnight at **4°C**.
20. Rinse off slides with Tris.

21. Wash in running tap H_2O for **5 min**.
22. Wash in Tris for **5 min** in green boats.
23. Coverslip using non-photobleaching reagent (Prolong Gold with DAPI or FluorMount with DAPI). Allow to dry completely before imaging on scanner.

Solutions and Reagents

0.5 M Tris (8 L)

Needed (mL)	Stock Solution	Final Concentration
5 L	dH_2O	
485 g	Tris base	0.5 M
240 mL	Concentrated HCl	
pH to 7.6		
To 8L	dH_2O	

Reagents

Vendor	Catalog #	Qty	Unit Price	Description
RNAscope® Multiplex Fluorescent Reagent Kit V2	323100		1330.00	Contains H2O2, protease reagents, target retrieval reagent, wash buffer, HRP reagents
RNAscope® 3-plex Positive Control Human	320861		100.00	Polr2a (C1 channel) and PPIB (C2 channel), UBC (C3 channel)
Sigma	199664-25G	1	66.60	Sudan Black B
Vector Laboratories	H-4000	1	120.00	ImmEdge Hydrophobic Barrier Pen
Southern Biotech	0100-01	1	45.14	DAPI Fluoromount-G

Protocol for harvesting and dissociating mouse brain neurons for single cell RNA Sequencing on the 10X Genomics platform.

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ABSTRACT

Single-cell RNA sequencing has emerged as a powerful method to characterize gene expression on a single cell level. Producing useful data with this method critically relies on obtaining a suspension of dissociated cells with high concentration and viability from the tissue of interest. This protocol allows to isolate and dissociate mouse brain cells into a concentrated cell suspension that is compatible with the 10X Genomics library preparation and sequencing pipeline and enables capturing up to 10,000 single cells.

Isolation of neurons is based on the following published protocol with minor modifications:

> Vazirani, R. P., Fioramonti, X., Routh, V. H. Membrane Potential Dye Imaging of Ventromedial Hypothalamus Neurons From Adult Mice to Study Glucose Sensing. J. Vis. Exp. (81), e50861, doi:10.3791/50861 (2013). https://www.jove.com/t/50861/membrane-potential-dye-imaging-ventromedial-hypothalamus-neurons-from

The protocol was further adapted for 10X Genomics platform according to the 10X Genomics "Cell Preparation Guide" (CG00053 Rev C):

> https://support.10xgenomics.com/single-cell-gene-expression/sample-prep/doc/demonstrated-protocol-single-cell-protocols-cell-preparation-guide

Part 1: Advance preparation of solutions.

1. Prepare stock solutions of media supplements:
    1. Lactic acid: prepare 1M (90 mg/ml) solution of lactic acid in nuclease-free water and aliquot in 50 µl aliquots. Store at -20°C.
    2. GlutaMAX: aliquot original GlutaMAX (200 mM) solution into 100 µl aliquots. Store at -20°C.
    3. B27 (minus insulin) supplement: thaw original solution and aliquot into 700 µl aliquots. Store at -20°C.
2. Prepare 1L of Brain Perfusion Solution (composition: 2.5 mM KCl, 7 mM MgCl2, 1.25 mM NaH2PO4, 28 mM NaHCO3, 0.5 mM CaCl2, 7 mM glucose, 1 mM ascorbate, and 3 mM pyruvate

in nuclease-free water). Adjust the osmolarity to ~300 mOsm using approximately 67 g/L sucrose. Oxygenate by bubbling with 95% O2 /5% CO2 for 15 min and adjust the pH to 7.4. Filter with a filter unit. Aliquot in 200 ml aliquots (each experiment will require approximately 200 ml of perfusion solution). Aliquots can be stored at -20 °C for up to 2 months. The day before experiment: thaw an aliquot of the Brain Perfusion Solution at 4°C overnight.

We prepare the solution from the following specific components (for 1L solution):

#	Component	Final Conc. (mM)	MW (g/mol)	Solid weight (mg/1000 mL)
1	Sucrose	196	342.3	67000.0
2	KCl	2.5	74.55	186.4
3	NaHCO3	28	84.01	2352
4	NaH2PO4 * H20	1.25	137.99	172.5
5	Glucose	7	180.16	1261
6	Sodium Ascorbate	1	198.11	198.1
7	CaCl2 * 2H20	0.5	147.01	73.5 (or 0.5ml of 1 M stock)
8	MgCl2	7	95.21	666.5 (or 7ml of 1 M stock)
9	Sodium Pyruvate	3	110.04	30 ml of 100 mM stock

3. The day before experiment, prepare a fresh 30 ml aliquot of Hibernate A media with 2.5 mM glucose, osmolarity of 280 mOsm, and 100 U/ml penicillin-streptomycin.

   Note: target osmolarity of the working Hibernate A media is 280 mOsm. We order a custom formulation from BrainBits with 2.5 mM glucose and osmolarity nominally pre-adjusted to 275 mOsm. The actual measured osmolarity of the supplied media varies between ~270-280. Ordering it with the requested 275 mOsm allows to adjust up to the target 280 mOsm. After receiving a new batch of media, we measure the original osmolarity, calculate approximate amount of NaCl to add to reach 280 mOsm, validate this amount empirically, and use the same amount for every experiment with a given batch/bottle of media to consistently obtain a working solution with the required target osmolarity of 280 mOsm.

   1. In the sterile culture hood, transfer 30 ml of stock Hibernate A (pre-made by the supplier with 2.5 mM glucose and nominal osmolarity of 275 mOsm) to the 50 ml culture flask.
   2. Add 300 µl of 100X Pen-strep stock solution to 30 ml Hibernate A aliquot.
   3. Add NaCl (cell culture grade) to adjust osmolarity to 280 mOsm (~0-5 mg).
   4. Store media aliquot at 4°C in dark until the day of experiment.

4. Fire-polish the tips of eight 9-inch glass Pasteur pipettes: 4 pipettes barely polished until the tips are no longer sharp (for cell transfers) and 4 pipettes for trituration with the following approximate tip opening diameters: 0.9mm; 0.7mm; 0.5mm; 0.3mm. The largest should be barely polished and the smallest should be about a third of that diameter.

Part 2. Experimental procedure on the day of experiment.

1. Maintain RNAse-free conditions throughout the procedure. Wipe down all surfaces and tools with the "RNAse-away" reagent.
2. Thaw frozen aliquots of lactic acid, GlutaMax and B27 (minus insulin) supplements, vortex.

3. Prepare working solution of the Hibernate A media by adding the supplements for the following final working concentrations:
    i. Lactic acid: 1 mM (add 30 µl of 1M stock solution to the 30 ml media aliquot).
    ii. GlutaMax: 0.5 mM (add 75 µl of 200 mM stck solution to the 30 ml media aliquot) .
    iii. B27: 2% (add 600 µl of the stock solution to the 30 ml media aliquot).
4. Mix media aliquot by inversion. Adjust pH to 7.4 using 1N NaOH.
5. Oxygenate the thawed 200 ml aliquot of the Brain Perfusion Solution with 95% O2/5% CO2 on ice for at least 15 min.
6. Prepare a 50 ml culture flask labeled "digestion" and 9 polystyrene 15-ml conical tubes labeled "harvest", "dissection", "papain", "DNAse", "BSA", "BSA filtered", "BSA centrifugation", "trituration", "cell suspension".
7. Distribute Hibernate-A media: transfer 2 ml to the "harvest" tube, 9 ml to "dissection" tube, 4 ml to "papain" tube, 5.5 ml to "DNAse" tube; 5 ml to "trituration" tube. Preserve the rest of Hibernate A media for next steps.
8. Place "harvest" and "dissection" tubes with media on ice near the animal dissection area.
9. Prepare RNA-se free area for animal dissection: wipe all surfaces and tools with "RNAse Away" reagent.
10. Prepare vibratome: get a new blade, wash with 70% ethanol followed by ultrapure water, load the blade in the vibratome blade holder.
11. Prepare area and tools for cardiac perfusion and animal dissection: bath for cardiac perfusion with absorbent pad, surgical tools, cardiac perfusion system with an oxygenation line inserted into perfusion reservoir.
12. Fill cardiac perfusion system with ultrapure water and let it run through to clean.
13. Add isoflurane to induction chamber for animal anesthesia.
14. Fill outer vibratome bath with ice and install on the vibratome.
15. Place 2 glass Petri dishes on ice.
16. Prepare oxygenation line (with 95% O2/5% CO2) to be later inserted into inner vibratome bath.
17. Place a mouse into induction chamber with isoflurane and wait until it stops breathing.
18. Take mouse out and verify depth of anesthesia by absence of response to toe pinch.
19. Pour ~30 ml of the ice-cold oxygenated Brain Perfusion Solution into reservoir of cardiac perfusion system just prior to dissection, fill tubing, and stop when about 20 ml of solution is left in the reservoir to be used for perfusion. Continue oxygenating the rest of the Brain Perfusion Solution on ice.
20. Perform mouse dissection (note: the quicker cardiac perfusion is started, and then the brain extracted, the better for the survival of neurons): open abdominal cavity with scissors and peel back skin above ribcage, exposing diaphragm. Cut diaphragm and rib cage towards the forelimbs on both sides, then cut diaphragm along the edge of the rib cage to expose thoracic cavity. Be careful not to puncture the heart or big vessels. Peel back the ribcage towards the head, exposing the heart.
21. Insert the cardiac perfusion needle into left ventricle. Cut the right atrium with scissors.
22. Start the flow of perfusion system.
23. Perfuse about 15 ml of perfusion solution (~1 min) until the effluent is clear. Success of perfusion can be assessed by internal organs changing color to a lighter shade.
24. Fill a petri dish on ice with ~15 ml of Brain Perfusion Solution.
25. Decapitate the mouse, extract brain: cut skin above skull from caudal to rostral end and peel away to expose skull. Make a midline cut with scissors in the skull towards the eye sockets. Use fine Graefe forceps to break and peel pieces of skull away from midline, exposing the brain. Make a coronal cut with scissors between olfactory bulbs and the rest of the brain, and another cut

between the brain and spinal cord to mobilize the brain. Use a spatula to slightly lift the brain from the skull, use scissors to cut optical tracts, and finally extract the mobilized brain from the skull with a spatula (hippocampal tool) and push it into the Petri dish with the Brain Perfusion Solution.

26. Make a coronal cut between cerebellum and the rest of the brain, trying to make it as flat and perpendicular to the rostro-caudal axis of the brain as possible - this will make the brain sit flat on the vibratome stage and produce brain slices parallel to the coronal plane.
27. Lift the brain from the solution with a curved spatula and gently dab with filter paper to dry, especially the flat coronal aspect which will be glued down to the vibratome stage.
28. Put a little drop of super glue on the vibratome stage and spread it with a cotton tip to an area slightly larger than the brain.
29. Put the brain down on the stage area covered with glue, coronal aspect (now being the caudal end) down, rostral end up.
30. Mount the vibratome stage with the glued brain in the inner vibratome bath. About 10 seconds after the brain attachment, fill the vibratome bath with the remaining oxygenated Brain Perfusion Solution to completely cover the brain in the bath.
31. Rotate the stage of the vibratome with the brain to orient ventral (hypothalamic side) towards and dorsal cortex away from the blade.
32. Make sure the oxygenation line is inserted into vibratome bath and is turned on throughout the brain slicing.
33. Lower the vibratome blade into cutting position.
34. Using vibratome control pad, move the stage and blade as needed to perform the first cut close to the rostral end of the brain (facing up in the bath).
35. Start cutting 300-µm thick slices (vibratome settings: speed: 0.2 mm/sec, amplitude: 1 mm), observing the anatomical cues until the target area is reached. The level of bregma+1.0mm is reached approximately when left and right parts of corpus callosum meet in the middle.
36. After reaching the target area, cut two consecutive 300-µm thick slices to be collected.
37. Pour all 9 ml of Hibernate A media from the "dissection" tube into the second empty Petri dish on ice.
38. Transfer two target slices with a spatula from the vibratome bath into Petri dish with Hibernate A media.
39. Using 27G needles attached to 1 ml syringes used as cutting tools, dissect the target brain areas from slices. Further cut dissected pieces in two, to produce tissue pieces about 1x1 mm in size.
40. Collect tissue pieces with a glass pipette, transfer them to the "harvest" tube with Hibernate A media, and place the tube on ice.
41. Prepare digestion solution: add 80 U of stock papain suspension to the "papain" tube with 4 ml of media (for a final concentration of 20U/ml; calculate the volume of papain to get 80U beforehand based on the activity of specific batch, usually 65-85 µl). Mix by inversion and place in 34°C water bath. Check and mix by inversion every minute until the media is no longer cloudy (~4 min).
42. During "papain" incubation, place the "harvest" tube with tissue pieces into 34°C water bath.
43. Using 0.22µm syringe filter, filter papain solution into a "digestion" culture flask.
44. Using glass pipette, transfer tissue pieces from "harvest" tube into "digestion" culture flask with papain. Shake "digestion" flask to make sure tissue pieces are not clumped together but are floating separately to ensure proper digestion.
45. Incubate "digestion" flask in a shaking water bath at 34°C with shaking at 150 rpm for 30 min.
46. Meanwhile, prepare media solutions for trituration:
    i. Prepare DNAse solution: add 500 µl of Hibernate A media to a vial with DNAse solid, gently but thoroughly mix to dissolve completely (do not vortex, DNAse is sensitive to shear).

Transfer 500 µl of dissolved DNAse to the "DNAse" tube with 5.5 ml of Hibernate A media (final volume 6 ml, final concentration of DNAse 0.1 mg/ml). Invert gently to mix (do not vortex).

ii. Prepare BSA solution: weigh 160 mg of bovine serum albumin solid and add to the "bsa" tube with 2 ml of Hibernate A (final concentration: 8% BSA). Vortex for 30 sec. Using 0.22µm syringe filter, filter BSA solution into another "bsa filtered" tube. Transfer 1 ml of filtered BSA to the "bsa centrifugation" tube.

iii. Place 30 µm Miltenyi SmartStrainer onto "cell suspension" tube to collect and filter cell suspension.

47. When 30 min digestion is done, transfer tissue pieces from "digestion" to the "trituration" tube using glass pipette. Invert once and let tissue pieces settle. Aspirate almost all media with glass pipette, leaving only tissue pieces.
48. Add 3 ml of the trituration media ("DNAse" tube) to the "trituration" tube with tissue pieces. Triturate with the largest pipette (0.9 mm) 10 times (draw large volume to collect all tissue pieces with each trituration stroke) over approximately 30 sec. Wait 4 min for tissue pieces to settle. Use the second-largest (0.7 mm tip opening) glass pipette to transfer 2 ml from the top of the cell suspension onto the cell strainer on top of "cell suspension" tube.
49. Add 2 ml of trituration media to the "trituration" tube. Triturate 10 times with the second-largest 0.7 mm pipette. Wait 3 min for pieces to settle. Use the third (0.5mm) glass pipette to transfer the top 2 ml to the cell strainer/"cell suspension" tube.
50. Add 1 ml of trituration media to the "trituration" tube. Triturate 5 times with the third 0.5 mm pipette. Wait 2 min for tissue pieces to settle. Use the fourth 0.3 mm glass pipette to transfer all the remaining solution from the "trituration" to the cell strainer/"cell suspension" tube.
51. Remove cell strainer from the "cell suspension" tube and layer all the solution from this tube onto the BSA layer in the "BSA centrifugation" tube using a glass pipette. (Hold the target tube vertically, add drops gently and in a steady pace close to the solution surface to avoid disrupting the BSA layer and mixing solutions).
52. Centrifuge the "BSA centrifugation" tube 5 min at 1,000 rpm at RT. Using an Eppendorf 5810R swinging bucket centrifuge, this corresponds to 67 rcf. Set "break" setting to '0', i.e. no breaking.
53. After centrifugation is done, carefully aspirate almost all media from the tube, leaving about 50 µL of solution above the cell pellet.
54. Add 950 µl of Hibernate A media to cell pellet. Resuspend cells with the glass pipette (gently pipette up and down 10 times).
55. Transfer all (~1 ml) of the cell suspension from the "bsa centrifugation" tube to a new 2-ml Eppendorf LoBind tube.
56. Centrifuge at 300 rcf for 5 min in a small tabletop centrifuge (Eppendorf 5424: 1787 rpm) at room temperature.
57. Carefully remove supernatant to leave ~50 µl of the solution, avoiding disrupting the cell pellet (it won't be visible) and creating bubbles.
58. Using glass pipette, carefully resuspend the cell pellet in the remaining ~50 µl of solution (pipette up and down about 10 times, avoid creating bubbles). This is the final cell suspension used for 10X library preparation. Place and store tube on ice until starting the 10X single cell protocol.
59. To determine the volume of solution to use for library preparation, determine cell concentration by counting cells using a hemocytometer. Transfer a 10 µl aliquot from the final cell suspension to a new 2 ml LoBind tube. Add 10 µl of Trypan Blue stain. Mix gently with a pipette. Pipette 10 µl into a hemocytometer chamber. Count the number of live (transparent) and dead (blue) cells under the microscope. In case of harvesting primary and secondary motor areas from two 300-µm thick slices (4 tissue pieces ~1x2 mm in size), the expected cell concentration is ~1,000-2,000

cells/µl, expected viability is ~85%. To recover 10,000 cells in 10X protocol, the optimal concentration is 700-1,200 cells/µl. If obtained cell concentration is much higher than that, dilute cell suspension to the desired concentration by adding appropriate volume of Hibernate A media.

60. Proceed with the 10x Genomics Single Cell Protocol. (In our case, the samples are delivered to the Keck Biotechnology Resource Laboratory/Yale Center for Genome Analysis at Yale University for further processing).

Expected results:

In case of harvesting primary and secondary motor cortex areas from two 300-µm thick slices (4 tissue pieces ~1x2 mm in size), the expected cell concentration is ~1,000-2,000 cells/µl, expected viability is ~85%.

Figure 1 shows a representative image of the cell suspension loaded in hemocytometer for counting.

Please note that this image was captured for demonstration purposes ~30 min after cell isolation, which resulted in a much higher number of dead cells (stained blue) than fresh preparation to be used in an actual experiment for downstream processing. Otherwise, the image gives a general idea of how the isolated cell suspension looks like during the hemocytometer counting step. This preparation had a concentration of ~1,600 cells/µl and viability of ~81% when assessed immediately after isolation.

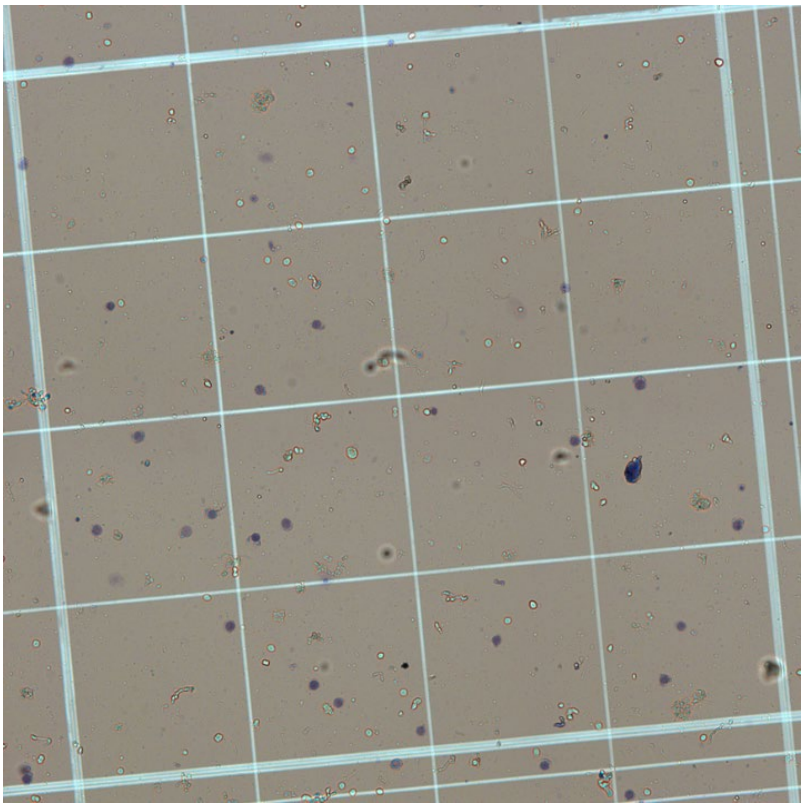

Figure 1: Microscopic image of the cell suspension loaded in the hemocytometer during the cell counting step.

Required custom equipment:

1. Oxygenation system, which consists of a gas tank with 95% O2/5% CO2 gas mixture and 3 tubing lines to provide continuous oxygenation and supply the gas to 1) a bottle with Brain Perfusion Solution, 2) reservoir (syringe) of the cardiac perfusion system, and 3) vibratome bath.
2. Cardiac perfusion system (gravity-driven), which consists of a 60-ml syringe secured ~65 cm above the working surface for mouse dissection with 1/4'' ID tubing (~1m long) ending with a Luer connection to a 20G needle. One of the oxygenation lines from the oxygenation system is inserted into the syringe reservoir of the perfusion system for oxygenation of the perfusion solution.

Materials:

Item	Supplier	Catalog No.
Brain perfusion solution		
Sucrose	Sigma	S0389
Potassium Chloride	JT Baker	3040-01
Sodium Bicarbonate	Sigma	S8875
Sodium Phosphate, Monobasic, Monohydrate	JT Baker	3818-01
Glucose	Sigma	G5767
(+)-Sodium L-ascorbate	Sigma	A4034
Calcium Chloride, Dihydrate	JT Baker	1332-01
Magnesium Chloride Anhydrous	Sigma	M8266
Sodium Pyruvate solution (100 mM)	ThermoFisher	11360-070
Sodium Chloride	Sigma	S5886
Media		
Hibernate A (custom order with 2.5 mM Glucose; osmolarity 275 mOsm)	BrainBits	CUSTOM-HA
Lactic acid	Sigma	L1750
GlutaMAX (100X)	ThermoFisher	35050-061
B27 Supplement minus insulin 50x, 10 ml	ThermoFisher	A1895601
Penicillin-Streptomycin (10,000 U/mL)	ThermoFisher	15140-122
Papain	Worthington	LS003124
DNAse	Worthington	LK003172
Bovine Serum Albumin	Sigma	A9418-5G
Labware		
Falcon 15 mL Polystyrene Centrifuge Tube	Corning	352099
Cell culture flask, 50 ml	Greiner	690160
10 ml Syringes	BD	309604
Millex-GS Syringe Filter Unit, 0.22 µm	EMD Millipore	SLGS033SS
Glass Pasteur Pipets	VWR	14673-043
DNA LoBind Tubes, 2.0 ml	Eppendorf	022431048
Nalgene Rapid-Flow Sterile Single Use Vacuum Filter Units, 1000 ml, 0.2 µm pore size	ThermoFisher	567-0020
PYREX 100x15 mm Petri Dish with Cover	Corning	3160-101
MACS SmartStrainers, 30 µm	Miltenyi Biotec	130-098-458
Equipment		

Vibratome	Leica	VT1200
Centrifuge	Eppendorf	5810 R
Centrifuge	Eppendorf	5424
Shaking Water Bath, 12L	VWR	10128-126
OsmoTECH Single-Sample Micro-Osmometer	Advanced Instruments	Osmotech
Bright-Line Hemocytometer	Sigma	Z359629
Surgical tools		
Surgical Scissors, Sharp-Blunt, Straight	Fine Science Tools	14001-18
Student Fine Scissors, Straight	Fine Science Tools	91460
Graefe Forceps, Straight	Fine Science Tools	11051-10
Hippocampal Tool (spatula)	Fine Science Tools	10099-15
Flat/Spoon Spatula, Stainless Steel	VWR	82027-532
Other reagents/supplies		
RNase AWAY Decontamination Reagent	ThermoFisher	10328011
Trypan Blue Stain (0.4%)	ThermoFisher	15250-061
Krazy Glue All Purpose Precision Tip	Elmer's Krazy Glue	
1 ml TB Syringe Slip Tip	BD	309659
PrecisionGlide Needle 27G x ½	BD	305109
PrecisionGlide Needle 20G x 1 ½	BD	305179
Filter Papers Whatman 1 55mm	GE Healthcare	1001-055
Double Edge Coated Blade Washed Version (for vibratome)	Electron Microscopy Sciences	72000-WA
Single Edge Industrial Razor Blade No.9	VWR	55411-050

References

1. Vazirani, R. P., Fioramonti, X., Routh, V. H. Membrane Potential Dye Imaging of Ventromedial Hypothalamus Neurons From Adult Mice to Study Glucose Sensing. J. Vis. Exp. (81), e50861, doi:10.3791/50861 (2013).
2. https://support.10xgenomics.com/single-cell-gene-expression/sample-prep/doc/demonstrated-protocol-single-cell-protocols-cell-preparation-guide

Safety warnings

Please follow the Safety Data Sheets (SDS) for all reagents for safe handling and safety hazards.