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

DOI

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**Manuscript citation:**

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-hypothalamusneurons-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/demonstratedprotocol-single-cell-protocols-cell-preparation-guide>

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Protocol status: Working

We use this protocol and it's working

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Keywords: single cell RNA Sequencing, dissociating mouse brain neurons, 10X Genomics platform, ASAPCRN, single cell rna, single cell, dissociating mouse brain neuron, single cell level, mouse brain neuron, dissociated cell, 10x genomics library preparation, 10x genomics platform, cell rna, compatible with the 10x genomics library preparation, concentrated cell suspension, cells with high concentration, mouse brain, neuron, sequencing pipeline

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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.

Attachments



[338-741.pdf](#)

527KB



Guidelines

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-hypothalamusneurons-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/demonstratedprotocol-single-cell-protocols-cell-preparation-guide>

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/demonstratedprotocol-single-cell-protocols-cell-preparation-guide>



Materials

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:

Brain perfusion solution

☒ Sucrose molecular biology grade **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S0389**

☒ Sodium bicarbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S8875**

☒ Glucose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G5767-500G**

☒ Sodium L-ascorbate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A4034**

☒ 1 M Magnesium Chloride (MgCl₂) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M8266**

☒ Sodium Pyruvate (100 mM) **Thermo Fisher Scientific Catalog #11360070**

☒ Sodium chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5886**

Media

☒ L-()-Lactic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #L1750**

☒ Glutamax (100x) **Gibco - Thermo Fisher Scientific Catalog #35050-061**

☒ B-27[®] Supplement, minus insulin **Thermo Fisher Catalog #A1895601**

☒ Penicillin/Streptomycin **Thermo Fisher Scientific Catalog #Invitrogen 15140-122**

☒ Bovine Serum Albumin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A9418**

Labware

Equipment	
15 mL Polystyrene Centrifuge Tube, Conical Bottom, with Dome Seal Screw Cap, Sterile, 50/Rack, 500/Case	NAME
Polystyrene Centrifuge Tube	TYPE
Falcon®	BRAND
352099	SKU
https://ecatalog.corning.com/life-sciences/b2c/US/en/Liquid-Handling/Tubes,-Liquid-Handling/Centrifuge-Tubes/Falcon%C2%AE-Conical-Centrifuge-Tubes/p/352099	LINK

Equipment	
CELL CULTURE FLASK, 50 ML, 25 CM², PS	NAME
CELL CULTURE FLASK	TYPE
CELLSTAR® TC	BRAND
690160	SKU
https://shop.gbo.com/en/india/products/bioscience/cell-culture-products/cellstar-cell-culture-flasks/standard-cell-culture-flasks/690160.html	LINK

 10cc syringe **Becton Dickinson (BD)** Catalog #309604

Equipment

Syringe Filter Unit, 0.22 µm, mixed cellulose esters, 33 mm, ethylene oxide sterilized

NAME

sterile syringe filter

TYPE

Millex-GS

BRAND

SLGS033SS

SKU

https://www.merckmillipore.com/IN/en/product/Millex-GS-Syringe-Filter-Unit-0.22m-mixed-cellulose-esters-33mm-ethylene-oxide-sterilized,MM_NF-SLGS033SS

LI
NK

Equipment

VWR® Disposable Pasteur Pipets, Glass

NAME

Disposable Pasteur Pipets

TYPE

VWR®

BRAND

14673-043

SKU

<https://us.vwr.com/store/product/4758176/vwr-disposable-pasteur-pipets-glass>

LINK

⊗ DNA LoBind Tube 2.0 mL **Eppendorf Catalog #022431048**

⊗ Nalgene®; Rapid-Flow®; Sterile Disposable Filter Units with PES Membrane, 1000mL, Pore Size: 0.20µm, 90mm membrane **Thermo Fisher Catalog #567-0020**

Equipment	
PYREX® 100×15 mm Petri Dish with Cover	NAME
Petri Dish with Cover	TYPE
PYREX®	BRAND
3160-101	SKU
https://ecatalog.corning.com/life-sciences/b2c/US/en/General-Labware/Dishes/Dishes,-Glass/PYREX%C2%AE-Petri-Dishes-with-Cover/p/3160-101	LIN K

 MACS SmartStrainers 30um **Miltenyi Biotec** Catalog #130-098-458

Equipment

Equipment	
Leica VT1200 - Semi-Automatic Vibrating Blade Microtome	NAME
Vibrating Blade Microtome	TYPE
Leica VT1200	BRAND
VT1200	SKU
https://www.leicabiosystems.com/histology-equipment/sliding-and-vibrating-blade-microtomes/vibrating-blade-microtomes/leica-vt1200/	LIN K

Equipment

Centrifuge 5810 R	NAME
Plate Centrifuge	TYPE
Eppendorf	BRAND
022625101	SKU

Equipment

Centrifuge	NAME
Bench centrifuge	TYPE
Eppendorf	BRAND
5424	SKU

Equipment

VWR® Shaking Water Baths	NAME
Shaking Water Baths	TYPE
VWR®	BRAND
10128-126	SKU
https://us.vwr.com/store/product/14446137/vwr-shaking-water-baths	LINK

Equipment	
OsmoTECH® Single-Sample Micro-Osmometer	NAME
Micro-Osmometer	TYPE
OsmoTECH®	BRAND
Osmotech	SKU
https://www.aicompanies.com/osmometers/osmotech-single-sample-micro-osmometer/ ^{LINK}	

Equipment	
Bright-Line™ Hemacytometer	NAME
Hemacytometer	TYPE
Bright-Line™	BRAND
Z359629	SKU
https://www.sigmaaldrich.com/US/en/product/sigma/z359629 ^{LINK}	

Surgical tools

Equipment	
Surgical Scissors - Sharp-Blunt	NAME
Surgical Scissors	TYPE
Surgical Scissors	BRAND
14001-18	SKU
https://www.finescience.com/en-US/Products/Scissors/Standard-Scissors/Surgical-Scissors-Sharp-Blunt/14001-18	LIN K

Equipment	
Student Fine Scissors Straight 11.5cm	NAME
Fine Scissors	TYPE
Student Fine	BRAND
91460-11	SKU
https://surgicaltools.co.uk/catalogue/student-instruments/fine-scissors-student-instruments/91460-11-student-fine-scissors-straight-11-5cm/	LIN K

Equipment	
Graefe Forceps	NAME
Forceps	TYPE
Graefe	BRAND
11051-10	SKU
https://www.finescience.com/en-US/Products/Forceps-Hemostats/Standard-Forceps/Graefe-Forceps/11051-10	LIN K

Equipment	
Hippocampal Tool	NAME
Tool	TYPE
Hippocampal	BRAND
10099-15	SKU
https://www.finescience.com/en-US/Products/Spatulae,-Spoons-Curettes/Hippocampal-Tools/Hippocampal-Tool/10099-15	LIN K

Equipment

VWR® Flat/Spoon Spatulas, Stainless Steel	NAME
Spatulas	TYPE
VWR®	BRAND
82027-532	SKU
https://us.vwr.com/store/product/4531759/vwr-flat-spoon-spatulas-stainless-steel	LINK

Other reagents/supplies

⊗ RNase AWAY™ Decontamination Reagent **Thermo Fisher Catalog #10328011**

⊗ Trypan Blue Solution, 0.4% **Thermo Fisher Catalog #15250061**

⊗ 1 ml tuberculin syringes **Becton Dickinson (BD) Catalog #REF 309659**

⊗ 27G needles **Catalog #305109**

⊗ Single-edge industrial razor blades **VWR International (Avantor) Catalog #55411-050**

	A	B	C
	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



A	B	C
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	22431048
Nalgene Rapid-Flow Sterile Single Use Vacuum Filter Units, 1000 ml, 0.2 µm pore size	ThermoFisher	567-0020
PYREX 100×15 mm Petri Dish with Cover	Corning	3160-101
MACS SmartStrainers, 30 µm	Miltenyi Biotec	130-098-458



A	B	C
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	Electron Microscopy	72000-WA

	A	B	C
	(for vibratome)	Sciences	
	Single Edge Industrial Razor Blade No.9	VWR	55411-050

Safety warnings

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

Ethics statement

Approval from an Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee should be obtained before performing these experiments. All animal procedures performed in relation to this protocol were performed in compliance with the Office of Animal Research Support of Yale University (protocol 2024-11497).

Part 1: Advance preparation of solutions

1 Prepare stock solutions of media supplements

1.1 **Lactic acid:** prepare 1 Mass Percent (90 mg/mL) solution of lactic acid in nuclease-free water and aliquot in 50 µL aliquots. Store at -20 °C .

1.2 **GlutaMAX:** aliquot original GlutaMAX (200 millimolar (mM)) solution into 100 µL aliquots. Store at -20 °C .

1.3 **B27 (minus insulin) supplement:** thaw original solution and aliquot into 700 µL aliquots. Store at -20 °C .

2 Prepare 1 L of Brain Perfusion Solution (composition: 2.5 millimolar (mM) KCl, 7 millimolar (mM) MgCl₂, 1.25 millimolar (mM) NaH₂PO₄, 28 millimolar (mM) NaHCO₃, 0.5 millimolar (mM) CaCl₂, 7 millimolar (mM) glucose, 1 millimolar (mM) ascorbate, and 3 millimolar (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 00:15:00 and adjust the pH 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 .

30m



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

	A	B	C	D	E
#	Component	Final Conc. (mM)	MW (g/mol)	Solid weight (mg/1000 mL)	
1	Sucrose	96	342.3	67000	

	A	B	C	D	E
2		KCl	2.5	74.55	186.4
3		NaHCO ₃	28	84.01	2352
4		NaH ₂ PO ₄ * H ₂ O	1.25	137.99	172.5
5		Glucose	7	180.16	1261
6		Sodium Ascorbate	1	198.11	198.1
7		CaCl ₂ * 2H ₂ O	0.5	147.01	73.5 (or 0.5ml of 1 M stock)
8		MgCl ₂	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 glucose, osmolarity of , and  100 U/ml penicillin-streptomycin.

Note

Note: target osmolarity of the working Hibernate A media is . We order a custom formulation from BrainBits with glucose and osmolarity nominally pre-adjusted to . The actual measured osmolarity of the supplied media varies between ~270-280. Ordering it with the requested allows to adjust up to the target . After receiving a new batch of media, we measure the original osmolarity, calculate approximate amount of NaCl to add to reach , 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 .



- 3.1 In the sterile culture hood, transfer  30 mL of stock Hibernate A (pre-made by the supplier with  2.5 millimolar (mM) glucose and nominal osmolarity of  275 mOsm) to the  50 mL culture flask.
- 3.2 Add  300 μ L of 100X Pen-strep stock solution to  30 mL Hibernate A aliquot. 
- 3.3 Add NaCl (cell culture grade) to adjust osmolarity to  280 mOsm (~  0 mg to  5 mg). 
- 3.4 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: 0.9 mm; 0.7 mm; 0.5 mm; 0.3 mm. 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.

- 5 Maintain RNase-free conditions throughout the procedure. Wipe down all surfaces and tools with the "RNase-away" reagent.
- 6 Thaw frozen aliquots of lactic acid, GlutaMax and B27 (minus insulin) supplements, vortex.
- 7 Prepare working solution of the Hibernate A media by adding the supplements for the following final working concentrations:
 - 7.1 **Lactic acid:**  1 millimolar (mM) (add  30 μ L of  1 Mass Percent stock solution to the  30 mL media aliquot). 
 - 7.2 **GlutaMax:**  0.5 millimolar (mM) (add  75 μ L of  200 millimolar (mM) stock solution to the  30 mL media aliquot) . 



- 7.3 **B27:** 2% (add  600 μ L of the stock solution to the  30 mL media aliquot). 
- 8 Mix media aliquot by inversion. Adjust pH to  7.4 using  1 Normality (N) NaOH. 
- 9 Oxygenate the thawed  200 mL aliquot of the Brain Perfusion Solution with 95% O₂/5% CO₂  On ice for at least  00:15:00 . 
- 10 Prepare a  50 mL culture flask labeled "digestion" and 9 polystyrene  50 mL conical tubes labeled "harvest", "dissection", "papain", "DNase", "BSA", "BSA filtered", "BSA centrifugation", "trituration", "cell suspension".
- 11 **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.
- 12 Place "harvest" and "dissection" tubes with media  On ice near the animal dissection area.
- 13 Prepare RNA-se free area for animal dissection: wipe all surfaces and tools with "RNase Away" reagent.
- 14 **Prepare vibratome:** get a new blade, wash with 70% ethanol followed by ultrapure water, load the blade in the vibratome blade holder.
- 15 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.
- 16 Fill cardiac perfusion system with ultrapure water and let it run through to clean.
- 17 Add isoflurane to induction chamber for animal anesthesia.
- 18 Fill outer vibratome bath with ice and install on the vibratome.



- 19 Place 2 glass Petri dishes On ice .
- 20 Prepare oxygenation line (with 95% O₂/5% CO₂) to be later inserted into inner vibratome bath.
- 21 Place a mouse into induction chamber with isoflurane and wait until it stops breathing.
- 22 Take mouse out and verify depth of anesthesia by absence of response to toe pinch.
- 23 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 .

- 24 Perform mouse dissection.

Note

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.

- 25 Insert the cardiac perfusion needle into left ventricle. Cut the right atrium with scissors.
- 26 Start the flow of perfusion system.
- 27 Perfuse about 15 mL of perfusion solution (~ 00:01:00) until the effluent is clear. Success of perfusion can be assessed by internal organs changing color to a lighter shade.

1m

28 Fill a petri dish  On ice with ~  15 mL of Brain Perfusion Solution.

29 **Decapitate the mouse, Extract brain:**

29.1 Cut skin above skull from caudal to rostral end and peel away to expose skull.

29.2 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.

29.3 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.

29.4 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.

30 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.

Note

This will make the brain sit flat on the vibratome stage and produce brain slices parallel to the coronal plane.

31 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.

32 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.

33 Put the brain down on the stage area covered with glue, coronal aspect (now being the caudal end) down, rostral end up.

34 Mount the vibratome stage with the glued brain in the inner vibratome bath. About  00:00:10 after the brain attachment, fill the vibratome bath with the remaining oxygenated Brain Perfusion Solution to completely cover the brain in the bath.

10s



- 35 Rotate the stage of the vibratome with the brain to orient ventral (hypothalamic side) towards and dorsal cortex away from the blade.
- 36 Make sure the oxygenation line is inserted into vibratome bath and is turned on throughout the brain slicing.
- 37 Lower the vibratome blade into cutting position.
- 38 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).
- 39 Start cutting $\rightarrow \pm 300 \mu\text{m}$ 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.
- 40 After reaching the target area, cut two consecutive $\rightarrow \pm 300 \mu\text{m}$ thick slices to be collected.
- 41 Pour all 9 mL of Hibernate A media from the "dissection" tube into the second empty Petri dish On ice .
- 42 Transfer two target slices with a spatula from the vibratome bath into Petri dish with Hibernate A media.
- 43 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 1×1 mm in size.
- 44 Collect tissue pieces with a glass pipette, transfer them to the "harvest" tube with Hibernate A media, and place the tube On ice .
- 45 **Prepare digestion solution:** add 80 U of stock papain suspension to the "papain" tube with 4 mL of media (for a final concentration of 20 U/ml ; calculate the volume of papain to get 80 U beforehand based on the activity of specific batch,

4m





usually $65\ \mu\text{L}$ to $85\ \mu\text{L}$). Mix by inversion and place in $34\ ^\circ\text{C}$ water bath. Check and mix by inversion every minute until the media is no longer cloudy (~ $00:04:00$).

46 During "papain" incubation, place the "harvest" tube with tissue pieces into $34\ ^\circ\text{C}$ water bath.

47 Using $0.22\text{-}\mu\text{m}$ syringe filter, filter papain solution into a "digestion" culture flask.

48 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.

49 Incubate "digestion" flask in a shaking water bath at $34\ ^\circ\text{C}$ with shaking at $150\ \text{rpm}$, $00:30:00$.



50 Meanwhile, prepare media solutions for trituration:

50.1 **Prepare DNase solution:** add $500\ \mu\text{L}$ 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 $500\ \mu\text{L}$ of dissolved DNase to the "DNase" tube with $5.5\ \text{mL}$ of Hibernate A media (final volume $6\ \text{mL}$, final concentration of DNase $0.1\ \text{mg/mL}$). Invert gently to mix (do not vortex).



50.2 **Prepare BSA solution:** weigh $160\ \text{mg}$ of bovine serum albumin solid and add to the "bsa" tube with $2\ \text{ml}$ of Hibernate A (final concentration: 8% BSA). Vortex for $30\ \text{sec}$. Using $0.22\ \mu\text{m}$ syringe filter, filter BSA solution into another "bsa filtered" tube. Transfer $1\ \text{mL}$ of filtered BSA to the "bsa centrifugation" tube.

50.3 Place $30\ \mu\text{m}$ Miltenyi SmartStrainer onto "cell suspension" tube to collect and filter cell suspension.

51 When $00:30:00$ digestion is done, transfer tissue pieces from "digestion" to the "trituration" tube using glass pipette. Invert once and let tissue pieces settle. Aspirate

30m



almost all media with glass pipette, leaving only tissue pieces.

52 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  00:00:30 . Wait  00:04:00 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.

4m 30s



53 Add  2 mL of trituration media to the "trituration" tube. Triturate 10 times with the second-largest 0.7 mm pipette. Wait  00:03:00 for pieces to settle. Use the third (0.5mm) glass pipette to transfer the top  2 mL to the cell strainer/"cell suspension" tube.

3m



54 Add  1 mL of trituration media to the "trituration" tube. Triturate 5 times with the third 0.5 mm pipette. Wait  00:02:00 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.

2m



55 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.



Note

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.

56 Centrifuge the "BSA centrifugation" tube  1000 rpm, Room temperature,  00:05:00 . Using an Eppendorf 5810R swinging bucket centrifuge, this corresponds to 67 rcf. Set "break" setting to '0', i.e. no breaking.

5m



57 After centrifugation is done, carefully aspirate almost all media from the tube, leaving about  50 µL of solution above the cell pellet.



- 58 Add  950 μL of Hibernate A media to cell pellet. Resuspend cells with the glass pipette (gently pipette up and down 10 times). 
- 59 Transfer all (~  1 mL) of the cell suspension from the "bsa centrifugation" tube to a new  2 mL Eppendorf LoBind tube.
- 60 Centrifuge at  300 rcf, 00:05:00 in a small tabletop centrifuge (Eppendorf 5424: 1787 rpm) at  Room temperature .  
- 61 Carefully remove supernatant to leave ~  50 μL of the solution, avoiding disrupting the cell pellet (it won't be visible) and creating bubbles.
- 62 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. 
- 63 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 $\sim 1 \times 2$ mm in size), the expected cell concentration is $\sim 1,000$ - $2,000$ cells/ μL , expected viability is $\sim 85\%$. 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.   
- 64 Proceed with the 10x Genomics Single Cell Protocol.

Note

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

- 65 In case of harvesting primary and secondary motor cortex areas from two  300 μm thick slices (4 tissue pieces $\sim 1 \times 2$ mm in size), the expected cell concentration is $\sim 1,000$ – $2,000$ cells/ μl , expected viability is $\sim 85\%$.
- 66 Figure 1 shows a representative image of the cell suspension loaded in hemocytometer for counting.

Note

Please note that this image was captured for demonstration purposes ~  00:30:00 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 $\sim 1,600$ cells/ μl and viability of $\sim 81\%$ when assessed immediately after isolation.

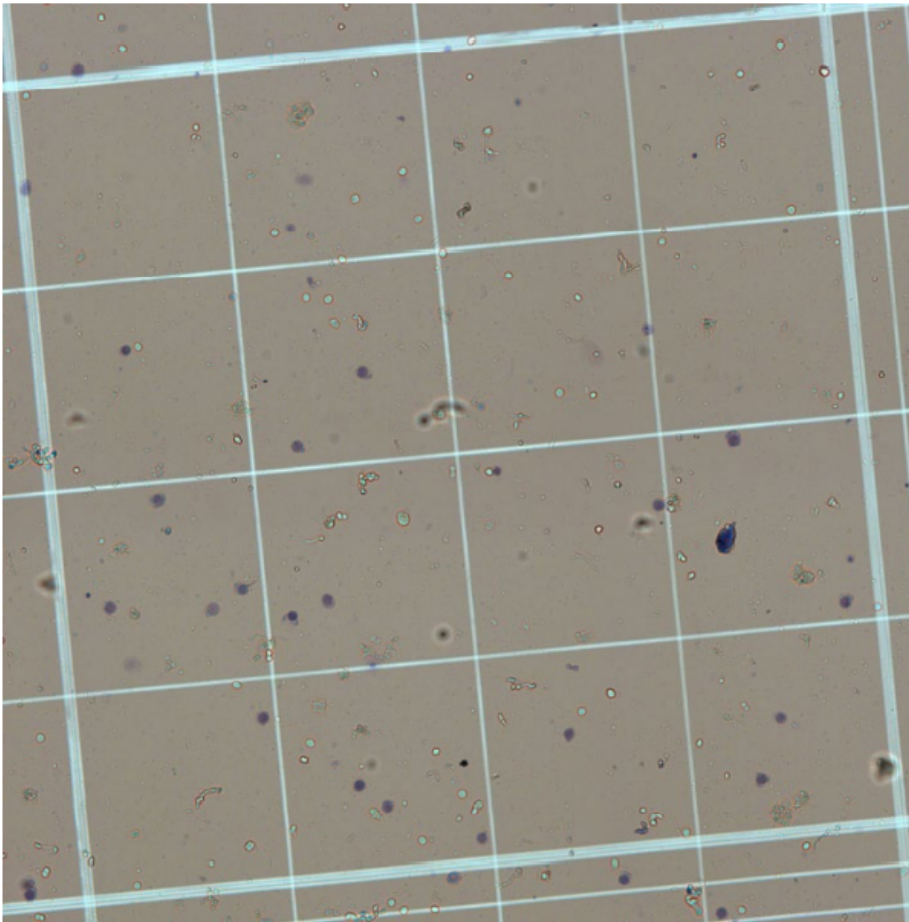


Figure 1: Microscopic image of the cell suspension loaded in the hemocytometer during the cell counting step.

Citation

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. Membrane Potential Dye Imaging of Ventromedial Hypothalamus Neurons From Adult Mice to Study Glucose Sensing.
J. Vis. Exp. (81), e50861,.

[10.3791/50861 \(2013\).](#)

[LINK](#)

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



Citations

Step 66

Vazirani, R. P., Fioramonti, X., Routh, V. H.. Membrane Potential Dye Imaging of Ventromedial Hypothalamus Neurons From Adult Mice to Study Glucose Sensing **10.3791/50861 (2013).**