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

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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

Protocol status: Working
We use this protocol and it's working

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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 cellsinto 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

PDF

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 **Sigma Aldrich Catalog #**S0389
- Sodium bicarbonate **Sigma Catalog #**S8875
- Glucose **Sigma Aldrich Catalog #**G5767-500G
- Sodium L-ascorbate **Sigma Aldrich Catalog #**A4034
- 1 M Magnesium Chloride (MgCl2) **Sigma Aldrich Catalog #**M8266
- Sodium Pyruvate (100 mM) **Thermo Fisher Scientific Catalog #**11360070
- Sodium chloride **Sigma Catalog #**S5886

Media

- L-( )-Lactic acid **Sigma Aldrich Catalog #**L1750
- Glutamax (100x) **Gibco - Thermo Fischer Catalog #**35050-061
- B-27™ Supplement, minus insulin **Thermo Fisher Catalog #**A1895601
- Penicillin/Streptomycin **Thermo Fisher Scientific Catalog #**Invitrogen 15140-122
- Bovine Serum Albumin **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 **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 LINK

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® 100x15 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

LINK

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/

LINK

Equipment

Centrifuge 5810 R NAME

Plate Centrifure 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	LINK

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/	LINK

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

LINK

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

LINK

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 **BD Biosciences Catalog #**REF 309659
- 27G needles **Catalog #**305109
- Single-edge industrial razor blades **Vwr 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
	Sodium Pyruvate solution (100 mM)	ThermoFisher	11360-070
	Sodium Chloride	Sigma	S5886
	Media		
	Hibernate A (custom order with 2.5 mM	BrainBits	CUSTOM-HA

	A	B	C
	Glucose; osmolarity 275 mOsm)		
	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 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

	A	B	C
	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

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 [M] 1 Molarity (M) (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 ([M] 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: [M] 2.5 millimolar (mM) KCl, [M] 7 millimolar (mM) $MgCl_2$, [M] 1.25 millimolar (mM) NaH_2PO_4, [M] 28 millimolar (mM) $NaHCO_3$, [M] 0.5 millimolar (mM) $CaCl_2$, [M] 7 millimolar (mM) glucose, [M] 1 millimolar (mM) ascorbate, and [M] 3 millimolar (mM) pyruvate in nuclease-free water). Adjust the osmolarity to ~ [M] 300 mOsm using approximately 67 g/L sucrose. Oxygenate by bubbling with 95% O_2 /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
	2	KCl	2.5	74.55	186.4
	3	NaHCO3	28	84.01	2352

	A	B	C	D	E
	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 [M] 50 millimolar (mM) glucose, osmolarity of [M] 280 mOsm, and 100 U/ml penicillin-streptomycin.

Note

Note: target osmolarity of the working Hibernate A media is [M] 280 mOsm. We order a custom formulation from BrainBits with [M] 50 millimolar (mM) glucose and osmolarity nominally pre-adjusted to [M] 275 mOsm. The actual measured osmolarity of the supplied media varies between ~270-280. Ordering it with the requested [M] 275 mOsm allows to adjust up to the target [M] 280 mOsm. After receiving a new batch of media, we measure the original osmolarity, calculate approximate amount of NaCl to add to reach [M] 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 [M] 280 mOsm.

3.1 In the sterile culture hood, transfer 30 mL of stock Hibernate A (pre-made by the supplier with [M] 2.5 millimolar (mM) glucose and nominal osmolarity of [M] 275 mOsm) to the 50 mL culture flask.

3.2 Add 300 µL of 100X Pen-strep stock solution to 30 mL Hibernate A aliquot.

3.3 Add NaCl (cell culture grade) to adjust osmolarity to [M] 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**: [M] 1 millimolar (mM) (add 30 µL of [M] 1 Molarity (M) stock solution to the 30 mL media aliquot).

7.2 **GlutaMax**: [M] 0.5 millimolar (mM) (add 75 µL of [M] 200 millimolar (mM) stck solution to the 30 mL media aliquot) .

7.3 **B27**: 2% (add 600 µL of the stock solution to the 30 mL media aliquot).

8 Mix media aliquot by inversion. Adjust pH to pH 7.4 using [M] 1 Normality (N) NaOH.

9 Oxygenate the thawed 200 mL aliquot of the Brain Perfusion Solution with 95% O_2/5% CO_2 On ice for at least 00:15:00 . 15m

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% O_2/5% CO_2) 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. 1m
Success of perfusion can be assessed by internal organs changing color to a lighter shade.

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 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.

40 After reaching the target area, cut two consecutive 300 µm 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 1x1 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, usually 65 µL to 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 (~ 00:04:00). 4m

46 During "papain" incubation, place the "harvest" tube with tissue pieces into 34 °C water bath.

47 Using 0.22-µm 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 °C with shaking at 150 rpm, 00:30:00 .

50 Meanwhile, prepare media solutions for trituration:

50.1 **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).

50.2 **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 0.22 µm syringe filter, filter BSA solution into another "bsa filtered" tube. Transfer 1 mL of filtered BSA to the "bsa centrifugation" tube.

50.3 Place 30 µm 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 almost all media with glass pipette, leaving only tissue pieces.

30m

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

4m 30s

pipette to transfer 2 mL from the top of the cell suspension onto the cell strainer on top of "cell suspension" tube.

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 . 5m

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 ~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.

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 ~1x2 mm in size), the expected cell concentration is ~1,000-2,000 cells/µl, expected viability is ~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 ~1,600 cells/µl and viability of ~81% when assessed immediately after isolation.

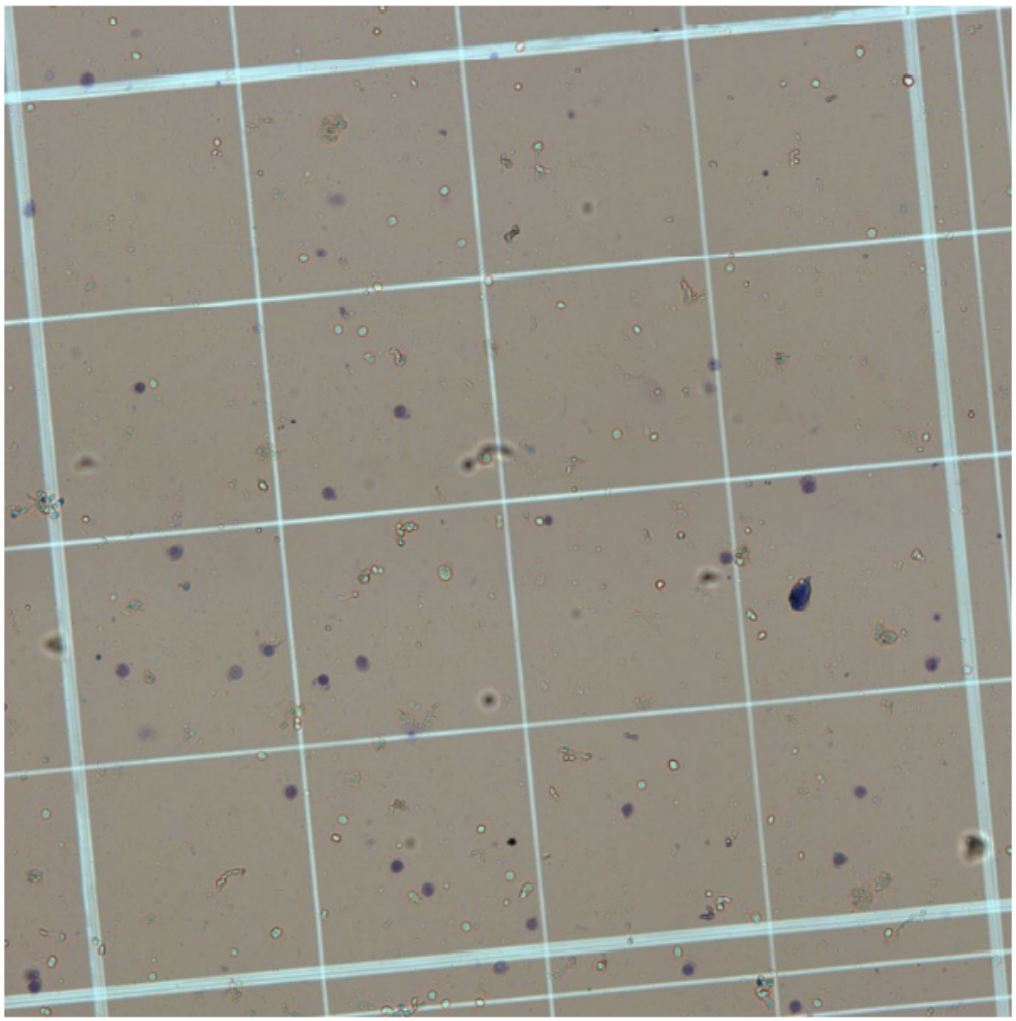

Figure 1: Microscopic image of the cell suspension loaded in the hemocytometer during the cell counting step.

CITATION

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,.
LINK
10.3791/50861 (2013).

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).