Title
Transcriptional profiling of mouse projection neurons with VECTORseq

Journal
STAR Protocols, 3(3)

ISSN
2666-1667

Authors
Cheung, Victoria
Chung, Philip
Feinberg, Evan H

Publication Date
2022-09-01

DOI
10.1016/j.xpro.2022.101625

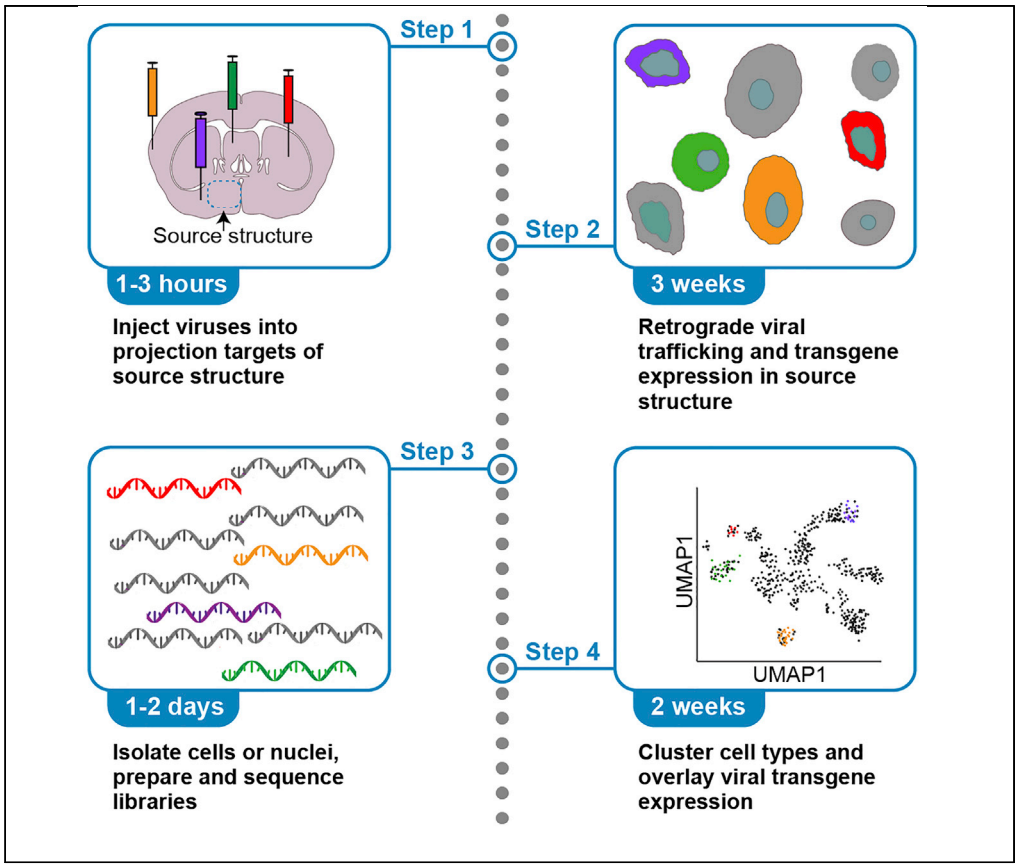
Peer reviewed

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Transcriptional profiling of mouse projection neurons with VECTORseq



Existing techniques for transcriptional profiling of projection neurons could be applied to only one neuronal population per experiment. To increase throughput, we developed VECTORseq, which repurposes retrogradely infecting viruses to deliver multiplexable RNA barcodes, enabling projection anatomy to be read out in single-cell datasets. In this protocol, we describe the delivery of viral barcodes to mouse brain to label different projection neurons. We then detail single-cell or nuclei isolation for sequencing, followed by the analysis of single-cell sequencing data.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Victoria Cheung, Philip Chung, Evan H. Feinberg

evan.feinberg@ucsf.edu

Highlights

Using VECTORseq for multiplexed transcriptional profiling of projection neurons

Strategies are provided for single-cell and single-nucleus sequencing

Analytical approaches for identifying viral barcodes in sequencing datasets

Cheung et al., STAR Protocols 3, 101625
September 16, 2022
https://doi.org/10.1016/j.xpro.2022.101625

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Transcriptional profiling of mouse projection neurons with VECTORseq

Victoria Cheung,[1,2,5] Philip Chung,[3] and Evan H. Feinberg[1,4,6,*]

[1]Department of Anatomy, University of California, San Francisco, San Francisco, CA 94158, USA

[2]Tetrad Graduate Program, University of California, San Francisco, San Francisco, CA 94158, USA

[3]Department of Anesthesiology & Pain Medicine, University of Washington, Seattle, WA 98195, USA

[4]Kavli Institute for Fundamental Neuroscience, University of California, San Francisco, San Francisco, CA 94158, USA

[5]Technical contact: victoriakcheung@gmail.com

[6]Lead contact

*Correspondence: evan.feinberg@ucsf.edu
https://doi.org/10.1016/j.xpro.2022.101625

SUMMARY

Existing techniques for transcriptional profiling of projection neurons could be applied to only one neuronal population per experiment. To increase throughput, we developed VECTORseq, which repurposes retrogradely infecting viruses to deliver multiplexable RNA barcodes, enabling projection anatomy to be read out in single-cell datasets. In this protocol, we describe the delivery of viral barcodes to mouse brain to label different projection neurons. We then detail single-cell or nuclei isolation for sequencing, followed by the analysis of single-cell sequencing data.

For complete details on the use and execution of this protocol, please refer to Cheung et al. (2021).

BEFORE YOU BEGIN

This protocol is used to prepare mice for stereotaxic surgeries and subsequently single-cell or single-nuclei isolation. However, it is likely that the VECTORseq approach could be applied to examine projection neurons in other species. This study was approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco. All animal studies must be approved by an Institutional Animal Care and Use Committee (IACUC) and performed in accordance with IACUC guidelines.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-NeuN conjugated to Alexa Fluor 488	Abcam	Cat# ab190195
Bacterial and virus strains		
AAVrg-Ef1α-mCherry-IRES-Cre	Addgene	Cat# 55632-AAVrg
AAVrg-Ef1α-FLPo	Addgene	Cat# 55637-AAVrg
AAVrg-hSyn-Dre	Addgene	Cat# 50363-AAVrg
AAV1-CAG-FLEX-tdTomato	Addgene	Cat# 28306-AAV1
Critical commercial assays		
Papain Dissociation System Protocol (includes Ovomucoid inhibitor, Earle's Balanced Salt solution, DNase)	Worthington Biochemical Corporation	Cat# LK003150

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
5′ v2 Library prep kit	10x Genomics	Cat# 1000265
Chromium Next GEM Chip K Single Cell Kit, 16 rxns	10x Genomics	Cat# 1000287
Chromium Next GEM Single Cell 5′ Library and Gel Bead Kit v1.1, 4 rxns	10x Genomics	Cat# 1000167
Chromium Next GEM Chip G Single Cell Kit, 16 rxns	10x Genomics	Cat# 1000127
Neuron isolation kit	Miltenyi	Cat# 130-126-603
Deposited data		
Raw data	Cheung et al. (2021)	NCBI GEO (GSE189907)
Analysis	Cheung et al. (2021)	https://github.com/vic-cheung/vectorseq https://doi.org/10.5281/zenodo.5703724
Experimental models: Organisms/strains		
Adult male or female (8–12 weeks) C57BL/6J *mus musculus*	The Jackson Laboratory	Cat# 000664
Software and algorithms		
Cellranger 6.0.0	Zheng et al. (2017)	http://10xgenomics.com/
Scanpy 1.7.2	Wolf et al. (2018)	https://github.com/theislab/scanpy
Scrublet 0.2.1	Wolock et al. (2019)	https://github.com/swolock/scrublet
Python 3.9	Python Software Foundation	https://www.python.org/downloads/
Chemicals, peptides, and recombinant proteins		
Triton X -100	Sigma-Aldrich	Cat# X100 CAS: 9002-93-1
Kollidon VA64	BASF Pharma	CAS: 25086-89-9
Nuclease-Free Water	HyClone Products	Cat# 82007-334 CAS: 7732-18-5
$MgCl_2$ (1 M stock)	Crescent Chemical Co Inc	Cat# 50248458
Glucose	Supelco Inc	Cat# NC0651806
K_2So_4	Alfa Aesar	Cat# AAA139750B CAS: 7778-80-5
Na_2SO_4	Chem-Impex Intl Inc	Cat# 50491082 CAS: 7757-82-6
RNAse Inhibitor Lucigen	Lucigen Corporation	Cat# NC1819281
HEPES	MilliporeSigma	Cat# 80502-686 CAS: 7365-45-9
$MgSo_4$	Sigma-Aldrich	Cat# 746452 CAS: 7487-88-9
$CaCl_2$ (1 M stock)	Sigma-Aldrich	Cat# 21115-100ML CAS: 10043-52-4
$CaCl_2$ (powder)	Fisher Scientific	Cat# S75069 CAS: 10035-04-8
Pyruvic Acid	Sigma-Aldrich	Cat# 107360 CAS: 127-17-3
Ascorbic Acid	Sigma-Aldrich	Cat# 795437 CAS: 50-81-7
NMDG	Sigma-Aldrich	Cat# M2004 CAS: 6284-40-8
HCl	Sigma-Aldrich	Cat# 320331 CAS: 7647-01-0
KCl	Tocris	Cat# 31471KG CAS: 7447-40-7
NaH_2PO_4	Research Products International Corp	Cat# 50489015 CAS: 10049-21-5
$NaHCO_3$	Aldon Corp SE	Cat# 470302-438 CAS: 144-55-8

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Kynurenic Acid (KYNA)	Tocris	Cat# 3694100R CAS: 196901
DAPI	Thermo Fisher Scientific	Cat# 62248
autoMACS Rinsing Solution	Miltenyi	Cat# 130-091-222
Magnetic-Activated Cell Sorting Bovine Serum Albumin (MACS BSA) Stock Solution	Miltenyi	Cat# 130-091-376
Red Blood Cell Lysis Solution (10×)	Miltenyi	Cat# 130-094-183
NaCl	Neta Scientific Inc.	Cat# 7647-14-5
Carbogen	Airgas	Z020X9512000033
Other		
Model 940 Small Animal Stereotaxic Instrument	Kopf	N/A
UMP3 UltraMicroPump	World Precision Instruments	UMP3-1
Hand Warmer, 2-1/4 In. X 3-1/2 In.	HotHands	26KF14
Streile drapes	Henry Schein	9004686
Nair Hair Remover	Church & Dwight	22339
BD Aria II FACS Sorter	BD Biosciences	N/A
Vibratome	Leica	Cat# VT1200s
NovaSeq 6000	Illumina	N/A
-20°C freezer	SCI	Cat# SCGP21OW1AF
Spring Scissors – 8 mm Cutting Edge, Straight, Sharp	World Precision Instruments	Cat# 15024-10
Large Scissors for Decapitation; Blunt 6.5 cm scissors	VWR	Cat# 82027-594
Curved Dissecting Forceps	Fisher Scientific	Cat# S08097
Stainless Steel 19 cm Spoon	VWR	Cat# 89259-968
Razor Blade	Uline	Cat# 130013
Disposable Vacuum Filter/Storage Systems, Pore Size: 0.2um, Membrane Material: Cellulose Acetate, Volume: 1000mL	Corning	Cat# 976140
Filter Paper – 90 mm diameter	Whatman	Cat# 1001-090
Falcon Bacteriological Petri Dishes with Lid; 100 × 15mm	Falcon	Cat# 08757100D
Super Glue Gel Control	Loctite	Cat# 234790
Safety syringe, fixed needle, 1 mL 26G, 5/8" length	Thomas Scientific	Cat# 1145H04 (BX/1)
Gas Dispersion Tubes	Pyrex	Cat# 39533-12C
96-Well PCR Plate Block for use with Compact Dry Blocks	Thermo Scientific	Cat# EW-36403-44
Falcon 50 mL Conical Centrifuge Tubes	Falcon	Cat# 1495949A
Falcon 15 mL Conical Centrifuge Tubes	Falcon	Cat# 1495953A
0.5 mL Microcentrifuge Tube	Corning Axygen	Cat# 14222292
2 mL Low-Retention Microcentrifuge Tube	Fisher	Cat# 02681321
12-Well Cell Culture Plate	Corning Costar	Cat# 0720082
40 Micron Cell Strainer	Fisher Scientific	Cat# 22363547
Hemocytometer	INCYTO	Cat# DHCN015
Mini Centrifuge	Eppendorf AG 5424	Cat# 22620401
Swing Bucket Centrifuge	Sorvall ST16	Cat# 75004240
VWR Rocking platform Model 200	VWR	Cat# 40000-304
VWR Digital Water Bath Model 5L	VWR	Cat# 97025-130
2 mL Tubes Low-Retention	Fisher Scientific	Cat# 2681321
LS Columns and 13 mL tubes set	Miltenyi	Cat# 130-122-729
MidiMACS™ Separator	Miltenyi	Cat# 130-042-302
MACS MultiStand	Miltenyi	Cat# 130-042-303
Pre-Separation Filters	Miltenyi	Cat# 130-095-823
MACS Acrylic Tube Rack	Miltenyi	Cat# 130-041-406

MATERIALS AND EQUIPMENT

10x ACSF stock (1 L)		
Item	Concentration	Amount
MilliQ H_2O (0.22 μm filtered deionized H_2O)		800 mL
NaCl	1.2 M	70.1 g
KCl	30 mM	3.73 g
$NaHCO_3$	260 mM	21.84 g
NaH_2PO_4 (monobasic monohydrate)	12.5 mM	1.72 g

Note: Vacuum filter solution with 0.22 μm filter into a sterile 1 L container prior to use/for storage. (Corning Vacuum Filter in the KRT). Store at 4°C. Discard solution if it becomes cloudy.

1 M $MgSO_4$	
Item	Amount
$MgSO_4$	6.08 g
MilliQ H_2O	50 mL

Note: Store at room temperature (~20°C). Discard solution if it becomes cloudy.

1 M $CaCl_2$	
Item	Amount
$CaCl_2$	5.55 g
MilliQ H_2O	50 mL

Note: Or use 1 M stock solution listed in KRT—stored at room temperature. Discard solution if it becomes cloudy.

1x ACSF (1 L)		
Item	Concentration	Amount
10x ACSF Stock (above)		100 mL
Glucose	10 mM	1.8 g
1 M $MgSO_4$	2 mM	2 mL
1 M $CaCl_2$	2 mM	2 mL
Pyruvic Acid	0.1 mM	10 mg
Ascorbic Acid	0.4 mM	70 mg
MilliQ H_2O		Dilute up to 1 L

Note: Vacuum filter solution with 0.22 μm filter into a sterile 1 L container prior to use/for storage. (Corning Vacuum Filter in the KRT). Store at 4°C. Discard solution if it becomes cloudy. Do not store for more than 7 days.

NMDG buffer solution (1 L)		
Item	Concentration	Amount
NMDG	110 mM	21.47g
13 N HCl (stock concentration)	110 mM	8.46 mL
KCl	10 mM	2.03 g
NaH_2PO_4 (monobasic monohydrite)	1 mM	14.2 mg
1 M stock $CaCl_2$	.5 mM	0.5 mL

(*Continued on next page*)

Continued		
Item	Concentration	Amount
Glucose	25 mM	4.5 g
Pyruvic Acid (98% liquid)	3 mM	26.4 mg
Ascorbic Acid	10 mM	1.76 g
$NaHCO_3$	25 mM	2.1 g
MilliQ H_2O		Dilute up to 1 L

Note: Vacuum filter solution with 0.22 μm filter into a sterile 1 L container prior to use/for storage. (Corning Vacuum Filter in the KRT). Store at 4°C. Discard solution if it becomes cloudy. Do not store for more than 7 days.

Density Gradient Solution (All reagents are included in the Worthington Kit along with instructions on how to prepare required solutions)		
Item	Concentration	Amount
EBSS		2.7 mL
Ovomucoid Inhibitor	1 mg/mL	300 μL
Dnase	100 units/mL	150 μL
2 mM Kynurenic Acid (KYNA)	2 μm	3 μL
Store at 4°C.		

Dissociation Buffer (DB)		
Reagent	Concentration	Amount
Na_2SO_4	82 mM	5.83 g
K_2SO_4	30 mM	2.615 g
Glucose	10 mM	0.905 g
HEPES	10 mM	1.2 g
1 M $MgCl_2$	2.5 mM	2.5 mL
Nuclease-Free Water		500 mL
Store at 4°C.		

Extraction Buffer (ExB)		
Reagent	Concentration	Amount
DB		15 mL
Kollidon VA64	10 mg/mL	150 mg
Triton X-100 (1.7M)	1%	150 μL
10% BSA	0.01%	15 μL
RNase inhibitor, Lucigen, 40,000 units/mL	667 units/mL	250 μL
Store at 4°C.		

Note: The other components can be mixed in advance but add the RNase inhibitor at the last moment.

Wash Buffer (WB)		
Reagent	Concentration	Amount
DB		30 mL
10% BSA	0.01%	30 μL
RNase inhibitor, Lucigen, 40,000 units/mL	27 units/mL	20 μL
Store at 4°C.		

Note: The other components can be mixed in advance but add the RNase inhibitor at the last moment.

FACS Capture Buffer (CDB)		
Reagent	Concentration	Amount
DB		20 mL
10% BSA	0.01%	20 µL
Store at 4°C.		

FACS Buffer		
Reagent	Concentration	Amount
10% BSA stock solution	0.0025%	10 µL
autoMACS Rinsing Solution		40 mL
HEPES	25 mM	0.3g
RNase inhibitor, Lucigen, 40,000 units/mL	35 units/mL	35 µL
Store at 4°C.		

Note: The other components can be mixed in advance but add the RNase inhibitor at the last moment.

STEP-BY-STEP METHOD DETAILS

Stereotaxic injections

Timing: 2–3 weeks

This step delivers the viral barcodes to label different projection neurons. It is important to ensure that the retrograde viruses used infect the intended cell types.

Surgery: 1–5 h/mouse for surgeries

Passive wait time: 2–3 weeks for virus to traffic and express in source location

1. Administer preoperative analgesics according to approved IACUC protocol.
2. Prepare:
   a. Viral aliquot(s) in styrofoam ice box.
   b. Microinjection system.
   c. Scalpel blade.
   d. Biohazard bag for biohazard disposal.
   e. Heating pad.
   f. Sterile drapes to place on stereotaxic frame and over mouse.
   g. Sterilize all surgical tools in the bead sterilizer for approximately 10 s.
      i. Place on sterile drape.
   h. Sterile gloves.
   i. Hair removal agent(s).
3. Place the mouse in the stereotaxic frame, remove the hair from the scalp, make an incision through the scalp, and level the head in the frame.
4. Inject each projection target with a retrograde virus encoding a different transgene (Figure 1).

   Note: To reduce the likelihood of spillover and increase the efficiency of labeling, try to choose stereotaxic coordinates near the center of each target structure.

5. Close incision, recover mice from anesthesia, and return to home cage.

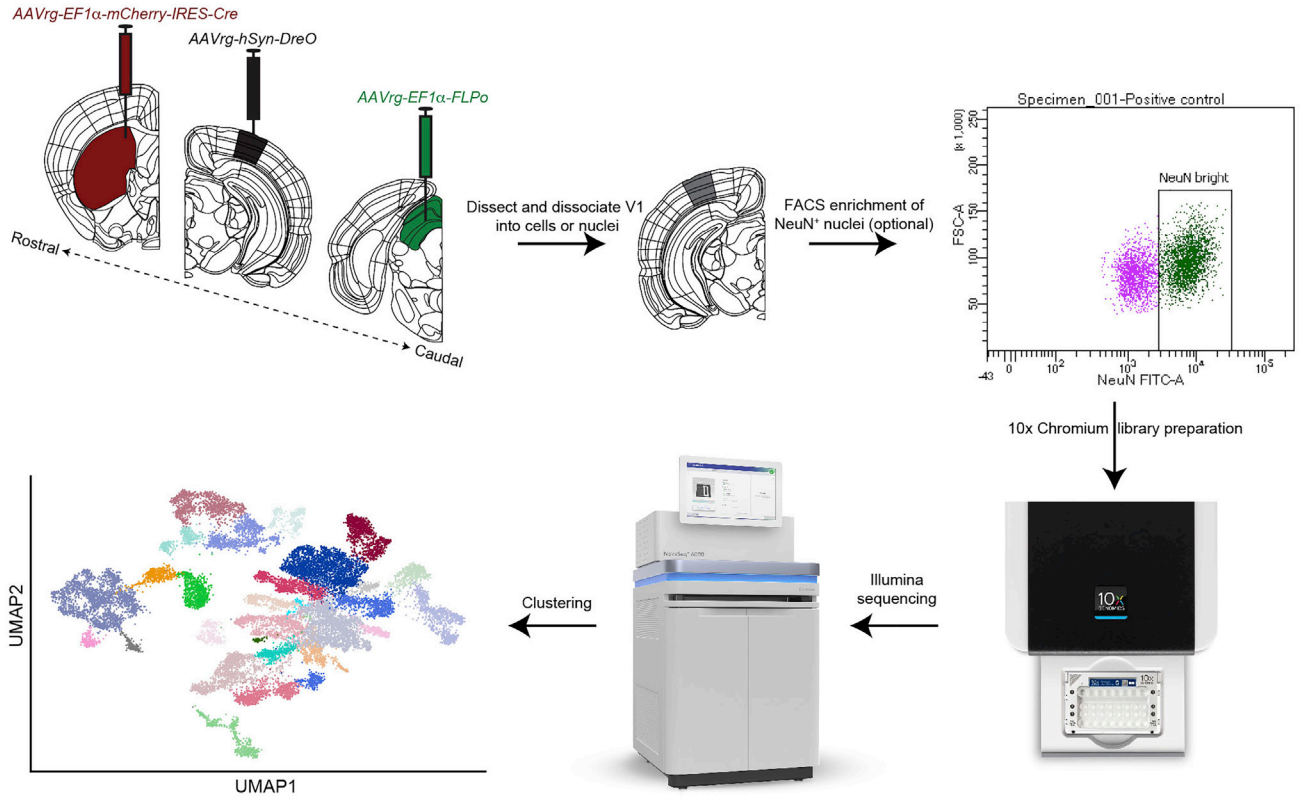


Figure 1. VECTORseq workflow

Retrogradely infecting viruses encoding different transgenes are injected into projection targets of a source structure of interest (in this schematic, primary visual cortex (V1)). Weeks later, the source structure is microdissected, dissociated into single cells or single nuclei (in an optional step, FACS enrichment of neuronal nuclei may be possible using expression of the neuronal marker NeuN, although note that not all neurons express NeuN), and single-cell or -nucleus libraries are prepared and sequenced. The sequencing datasets are then analyzed and viral transgene expression overlaid on clusters to identify projection populations.

Tissue slice preparation

Timing: 30 min–1 h

⚠ CRITICAL: Make sure all surfaces and tools are free of fixative.

This step slices the brain to enable subsequent microdissection of the regions of interest.

Pre-steps:

6. Prepare artificial cerebrospinal fluid (ACSF) and N-Methyl-D-glucamine (NMDG) buffer solution before starting.
   a. The NMDG solution used with the vibratome should be put in a -20°C freezer and shaken every 10–15 min to prevent clumping. Freeze and shake until you achieve a slurry/slushy consistency. This step will take approximately 1 h.
   b. Warm approximately 200 mL of NMDG in a glass dish set inside a warm 32°C–34°C water bath. Place a cell strainer into the dish of NMDG.

Note: Before use, take care to bubble the warming NMDG solution with carbogen for at least 30 min by a gas diffusion tube connected to a carbogen source directly into the solution (Figure 2B).

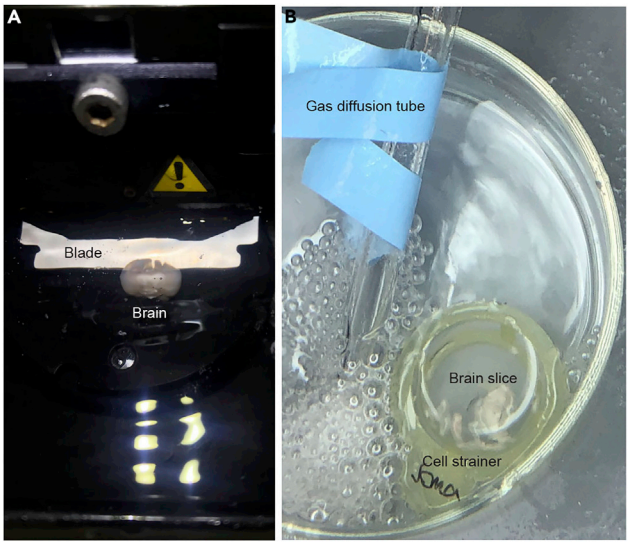


Figure 2. Tissue sectioning and incubation

(A) Illustration of brain on vibratome for sectioning. Coronal orientation. Brain is roughly 10 mm wide.

(B) Illustration of setup for incubating brain in carbogen-bubbled 1x ACSF.

7. Prepare the station in which you will sacrifice the mouse and retrieve the brain.
   a. Biohazard bag.
      i. Dissection tools: Large decapitation scissors, small spring scissors, forceps, spatula, and razor blade.
8. Deeply anesthetize the mouse to euthanize according to IACUC guidelines.
9. Transcardially perfuse mice with cold (4°C) 1x ACSF using gravity perfusion (approximately 5 min per mouse).
   a. It takes approximately 15 mL of 1x ACSF to perfuse one mouse.
10. Decapitate with large scissors.
    a. Quickly but carefully explant entire the brain. We used small spring scissors, forceps, and spatula for this step.
    b. Immediately place into NMDG slurry and wait 30 s.
11. Place a filter paper (e.g., a 90 mm Whatman filter disc) on a clean surface (e.g., a Petri dish).
    a. Remove the brain from the slurry and place directly onto the filter.
    b. Create a flat surface on the coronal plane by cutting the end of the brain that is not needed. We used a razor blade.

    Note: We cut off brainstem/cerebellum and adhered the brain directly onto the vibratome platform using Loctite glue with the olfactory bulb pointing up and our ROI oriented closer to the blade. Depending on the region of interest, the brain may need to be oriented differently.

    c. Orient the brain on the platform such that your region of interest is closest to the blade.
       i. We used the Paxinos mouse brain atlas as a guide to identify landmarks close to our ROI.
12. Cut 300 μm vibratome sections (Figure 2A).
    a. Use high speeds initially (e.g., 0.3 mm/s) to quickly trim away sections that do not contain areas that will be sequenced and slower speeds (e.g., 0.12 mm/s) when cutting sections containing the regions to be sequenced.
13. Recover slices in warm 32°C–34°C NMDG solution in the water bath. Continue bubbling with carbogen for 30–45 min.

Note: Use cell strainers floating in the NMDG dish to suspend slices (Figure 2B).

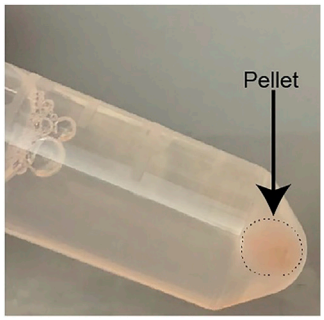


Figure 3. Example cell pellet in 2 mL microcentrifuge tube
This pellet is large and was subsequently resuspended and divided across two tubes.

a. While slices are recovering, prepare solutions for single-cell isolation or single-nuclei isolation.

14. Move slices into RT ACSF bubbled with carbogen. Slices can be kept alive up to 16 h.
15. Microdissect the region of interest under a dissection scope.

Single-cell isolation

Timing: 1.5–2 h

This step microdissects brain regions of interest and isolates single cells for sequencing.

Note: Prepare Worthington Papain Dissociation Kit + EBSS without Phenol Red, Miltenyi Adult Neuron Isolation Kit, 1x ACSF.

TISSUE DISSOCIATION AND FILTRATION OF CELLS –modified Worthington protocol

16. Transfer one slice at a time to a Petri dish and dissect region of interest from vibratome sections and return to ACSF. Once all slices have been microdissected, place regions of interest in a 2 mL low-retention centrifuge tube.
17. Follow the Papain Dissociation System Protocol (https://worthington-biochem.com/PDS/) from *Worthington Biochemical Corporation* to make the different solutions. Some steps are summarized below.
    a. Add 32 mL of Earle's Balanced Salt Solution (EBSS) to the albumin-ovomucoid inhibitor mixture. Can be stored at 2°C–8°C after reconstitution. Discard if there is discoloration or cloudiness that could indicate microbial growth.
    b. Add 5 mL of EBSS to the papain vial. Place papain in a 37°C water bath for 10 min until dissolved.
    c. Add 500 µL EBSS to DNAse vial, mix gently to prevent shear denaturation.
        i. Add 250 µL of this solution to the vial containing papain.
    d. Place tissue in the papain solution.
18. Incubate at 37°C with agitation for 1–1.5 h triturating with a transfer pipette or 200 µL pipette after 20 min and every 10 min thereafter. Be very gentle.
19. Once the dissociation is complete, centrifuge at:
    a. 300–350 *g* for 5–10 min, depending on the tissue.
        i. Timing should be tested on different ROIs. We recommend spinning at 7 min as a start and adjusting the timing from there.
    b. Meanwhile, prepare the **density gradient solution** (step 9 of protocol summary for Papain Dissociation System).
20. Discard the supernatant and prepare the density gradient.

a. DENSITY GRADIENT
   i. Add 500 μL of the EBSS-reconstituted albumin-ovomucoid inhibitor mixture into a micro-centrifuge tube.

Note: If the cell pellet after centrifugation is large, (example shown in Figure 3), fill more tubes since the goal is to maximize the number of cells passing through the density gradient solution. In most cases (e.g., isolating cells from a ~1 mm^3 structure isolated from 3-5 mice) 1–2 tubes should suffice.

   ii. Add 300 μL of **density gradient solution** to the cell pellet to resuspend and layer carefully on top of the 500 μL of EBSS-reconstituted albumin-ovomucoid to form a gradient. Do not mix.

Note: if the cell resuspension appears viscous (noticeably more viscous than water, similar to maple syrup) or opaque after adding the initial 300 μL of the density gradient solution, or if the cell pellet was large (Figure 3), add another 300 μL of the density gradient solution and fill another microcentrifuge tube with 500 μL of the EBSS-reconstituted albumin-ovomucoid inhibitor mixture. The result will be 2 density gradient tubes. Perform step iii.

   iii. Centrifuge at 100 *g* for 5–10 min to pellet the cells while leaving debris in the supernatant. For example, we used 6 min for ventral midbrain and 7 min for primary visual cortex (V1) and superior colliculus (SC).

Note: Users should troubleshoot which spin time works best for their tissue type.

   iv. Aspirate and discard the supernatant and reconstitute the cell pellet in 1 mL of 1x ACSF.

21. Count the cells using a hemocytometer; using trypan blue to assess viability.

Note: We only did this for cortex in the published paper. At this stage, we had ~~10^6 cells and had 1.4*10^5 cells after filtering.

22. Magnetic Labeling and Separation with LS Column: Perform all steps on ice. Directions are adapted from the instructions in the kit.
    Neuron Isolation Kit Data Sheet
    Midi MACS Data Sheet
    LS Columns Data Sheet

Note the cell count from Step 21.

a. Centrifuge cell suspension at 300 *g* for 10 min. Discard the supernatant.
   i. During centrifugation, prepare **d-MACS Buffer** as follows: for each sample, make 10 mL of a 1:20 dilution of the MACS BSA Stock solution in Dulbecco's Phosphate Buffered Saline (DPBS).
   ii. Prepare **MACS Buffer** as follows: for each sample, make 5 mL of a 1:20 dilution of the MACS BSA Stock solution in autoMACS Rinsing Solution.

Note: Keep all solutions on ice. Always use freshly diluted buffer.

b. Add 80 μL of d-MACS buffer per 10^7 total cells to the cell pellet.
c. Add 20 μL of the Adult Non-Neuronal Cell Biotin-Antibody cocktail per 10^7 total cells.
d. Mix well but **DO NOT VORTEX.** Incubate for 5 min on ice.
e. Wash cells by adding 2 mL of MACS buffer per 10^7 cells and centrifuge at 300 *g* for 10 min. Discard the supernatant.
f. Add 80 μL of d-MACS buffer per 10^7 cells to the cell pellet.
g. Add 20 μL of Anti-Biotin Microbeads per 10^7 total cells.
h. Mix well by inverting the tube and **DO NOT VORTEX.** Incubate for 10 min on ice.

i. Adjust volume up to 500 µL for up to 10^7 cells using the buffer. For more cells, scale up buffer volume accordingly.
j. Proceed to magnetic separation.
    i. Place LS column in the magnetic field referred to in the LS Columns Data Sheet.
    ii. Wash column twice with 1 mL of MACS buffer. Discard the eluate.
    iii. Apply cell suspension to the column by passing through the pre-separation filters. Collect filtrate into the collection tube provided with the LS columns.
    iv. Wash column 2x with 1 mL of d-MACS. Collect filtrate into the collection tube provided with the LS columns.

Note: Do not use the plunger as this would remove the magnetically bound cells. Unbound cells are the neurons.

k. Transfer filtrate into a 2 mL microcentrifuge tube.
l. Pellet the cells at 300 *g* for 10 min using a microcentrifuge at 4°C and remove supernatant for single-cell isolation.
m. Resuspend cells in 1 mL of 1x ACSF and note the count using a hemocytometer.
    i. Concentrate or dilute cells as necessary for 10x library preparation.

Single-nuclei isolation

Timing: 3–4 days

Timing: 20–30 min to overnight for pre-steps

Timing: 45 min–1 h active time for nuclei isolation

Timing: 2.5–4 h for FACS

Timing: 5–20 min for nuclei counting

Timing: 1–2 days for library prep

This step isolates single nuclei from brain regions of interest for single-cell sequencing.

23. PRE-CHILL FOR AT LEAST 20 min at –20°C or OVERNIGHT at 4°C:

Note: "Precoated with 1% BSA" means that 1% BSA solution was used to coat the item and the excess fluid was removed. Refer to Martin et al. (2020)protocol for extra detail. 1% BSA was made by using 10% BSA stock diluted in the autoMACS Rinsing Solution.

Item	Quantity
26G syringe	1
96-well metal PCR plate	1
50 mL Falcon tube precoated with 1% BSA	1
50 mL Falcon tube	1
15 mL Falcon tube precoated with 1% BSA	2
0.5 mL Eppendorf microcentrifuge tubes precoated with 1% BSA	5
12-well cell culture plate coated with 1% BSA	1
40 µm cell strainer	1
2 mL Eppendorf microcentrifuge tube	1
Collection tubes with 20 µL of FACS Capture Buffer (CDB) each (tubes provided by FACS core at Gladstone)	2

24. Solutions to Prepare (Night before or morning of dissociation)

1x ACSF

Chill in −20°C freezer to cool quickly (but be sure not to freeze).

⚠ CRITICAL: All solutions need to be chilled to 4°C and kept on ice prior to use. All steps are performed on ice or in a 4°C walk-in except for cell counting on a hemocytometer.

25. NUCLEI ISOLATION (~45 min- 1 h) based on Martin et al. (2020) protocol and Krishnaswami et al. (2016)
    a. Add 1 mL ExB (Extraction Buffer) to tissue region of interest and deposit into a well of a 12-well plate.
    b. Perform four trituration sets.
        i. A single trituration set is defined as 20 times of trituration followed by a 2-min wait period on ice. Triturate slowly with a 1 mL pipette tip.

    ⚠ CRITICAL: Do not create bubbles.

        ii. Repeat trituration cycle a 5th time only if the dissociation does not appear complete (e.g., large visible chunks).
    c. Pass the entire volume twice through a 26 G needle into the same well.
    d. Transfer into the pre-coated and pre-chilled 50 mL Falcon tube.
        i. Add 30 mL of WB (Wash Buffer).
        ii. Split volume evenly into the 2 1% BSA pre-coated, pre-chilled 15 mL Falcon tubes.
        iii. Spin down at 600 *g* for 10 min at 4°C using a swinging bucket rotor.
        iv. Remove supernatant until only ~500 μL remains in each tube. Combine the samples (pooled volume = 1 mL).
    e. Pass the suspension through the pre-chilled 40 μm cell strainer and filter using only gravity. Do not plunge or apply pressure.
    f. Transfer to a chilled 2 mL Eppendorf tube.
    g. Count nuclei on a hemocytometer. Make sure the concentration is roughly 10^6 nuclei/mL.
        i. To attain this concentration range, dilute or concentrate nuclei with 1x ACSF. Nuclei can be concentrated by following the gentle centrifugation protocol outlined in step 25j, removing the supernatant, and then adding desired volume of 1x ACSF.
    h. Reserve some nuclei for FACS negative control before staining (minimum volume required is usually around 200 μL). Keep on ice.
    i. Stain remaining nuclei with rabbit anti-NeuN antibody conjugated to Alexa Fluor 488 (ab190195).

    Note: Use 0.1–10 μg/mL anti-NeuN Ab. A dilution series should be tested to optimize concentration. We found that for our ROIs, 0.1 μg/mL was sufficient.

        i. Incubate in the dark at 4 °C for 30–45 min on a **gentle rocker** or tube rotator.
        ii. Wash three times with 200 μL FACS buffer (total 600 μL) and gentle centrifugation (15–20 min total).
    j. GENTLE CENTRIFUGATION PROTOCOL:
        i. Place a BSA-coated, pre-chilled 0.5 mL tube in a 2.0 mL tube inside a centrifuge.
        ii. Add nuclei to the 0.5 mL tube. Use a maximum volume of 200 μL.
        iii. Centrifuge at 200 *g* for 1 min at 4°C.
        iv. After reaching a complete stop, centrifuge again at 200 *g* for 1 min at 4°C.

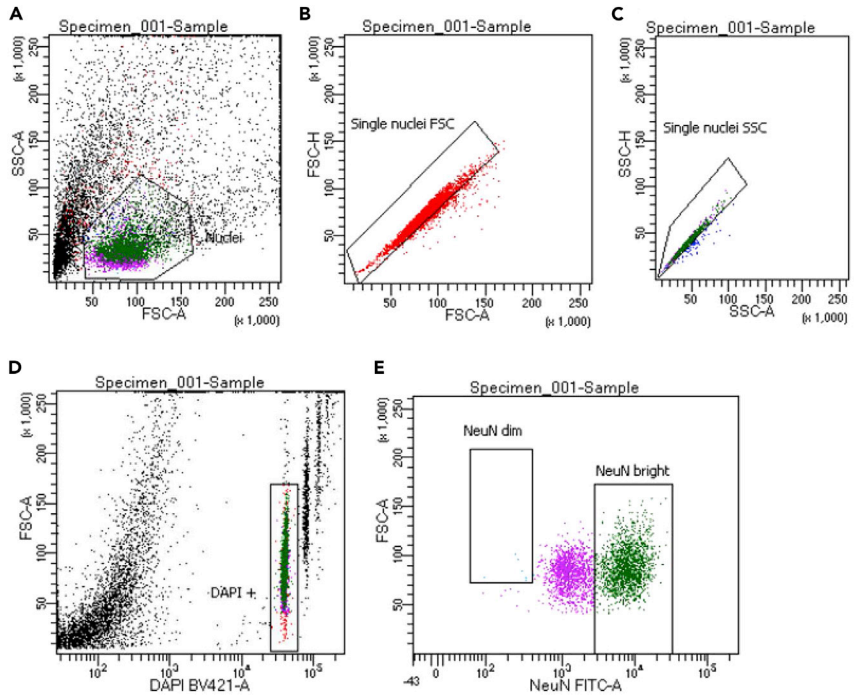


Figure 4. Example FACS plots for a single-nucleus experiment

(A) Gate for nuclei overlaid on forward scatter area (FSC-A) and side scatter area (SSC-A).

(B) Gate used for nuclei on plots of FSC-A against forward scatter height (FSC-H).

(C) as in B for side scatter.

(D) Gate for nuclei showing DAPI staining against forward scatter.

(E) Gate for NeuN+ nuclei.

v. Aspirate supernatant. Resuspend nuclei in FACS buffer at 10^7/mL, add DAPI at 1 mg/mL or 1:1000 dilution.

k. Proceed to FACS.

i. Bring the stained sample and the negative control (unstained) sample from step h.

ii. Fill collection tubes with 20 μL of FACS capture buffer (CDB) in each tube.

Note: Number of tubes are dependent on number of cells to be counted.

26. FACS (~1–4 h depending on sample size)

a. Use DAPI and anti-NeuN labeling to isolate neuronal nuclei (Figure 4). The NeuN labeling distribution is bimodal and we gate to include the peak with higher labeling.

b. To concentrate nuclei, perform GENTLE CENTRIFUGATION PROTOCOL described in step 25j. Centrifuge at 200 g for 1 min at 4°C.

27. COUNT NUCLEI AFTER FACS (~5 min–20 min).

a. Count nuclei on hemocytometer.

i. Prepare a 1:10 dilution of part of the sample (18 μL of chilled DB (Dissociation Buffer) with 2 μL of nuclei from the PCR tube).

ii. Load into hemocytometer.

iii. Examine fluorescent channels and the DAPI channel.

iv. Dilute nuclei with 1x ACSF or concentrate nuclei via gentle centrifugation protocol in a 4°C walk-in to 17,000 nuclei per 40 μL for the 10x Genomics library prep protocol. Proceed as soon as possible to 10x library preparation. Note: We have not waited longer than 45 min post-FACS (including counting).

28. LIBRARY PREP (1–2 days)
    a. Ensure that the reagents are shipped ahead of time.
    b. Prepare libraries using the 10x Genomics *Chromium single cell 5′ mRNA workflow* following standard protocol (https://support.10xgenomics.com/single-cell-gene-expression/sample-prep/doc/demonstrated-protocol-isolation-of-nuclei-for-single-cell-rna-sequencing-and-tissues-for-single-cell-rna-sequencing) and sequence libraries.
        i. Depending on the desired sequencing depth, we used either *NovaSeq* (2×10^{10} reads) or *NextSeq* ($1.3–2.6 \times 10^{8}$ reads). We aimed for 20–50,000 reads/cell and used standard paired-end 150 bp read lengths.

Genome alignment

Timing: Varies from 15 min–1 day depending on dataset size and hardware

This section describes methods used to align single-cell sequencing data with the reference genome and the addition of viral transgenes to the reference genome.

Raw gene sequence data outputs are converted to cell count tables using *Cell Ranger* provided by 10x Genomics.

29. Hardware: We recommend a computer with at least 8–16 CPU cores, 128 GB RAM, and 1–2 TB of disk storage depending on data size.
30. Operating System:
    a. Install a Debian-based Linux distribution such as Ubuntu or Centos. For our publication, we used Ubuntu on an Amazon Web Service (AWS) Elastic Compute Cloud (EC2) instance in our examples.
    b. Using the package manager on the Linux distribution, install tools needed to configure the python environment, including *wget, sh, bash.*
31. Cell Ranger:
    a. Visit the 10x Genomics download links here to download software and reference genomes.
    b. Download Cell Ranger (for our publication, we used version 6.0.0) software. We used the 10x Genomics *mm10* mouse reference genome.
    c. Follow instructions for building a custom reference genome here. Append custom reference information (such as viral transgenes) to the provided `.gtf` file within the reference genome. Then filter the `.gtf` file.
        i. create custom `.gtf` file

```
touch transgene.gtf
```

        ii. vim into the `.gtf` file and add in the transgene information. Example below.

```
transgene unknown exon 1 1443 . + . gene_id "transgene"; transcript_id "transgene"; gene_name "Transgene"
```

        iii. Append into the custom `transgene.gtf` file to the end of the provided `.gtf` file.

```
cat transgene.gtf >> genes.gtf
```

        iv. filter the `.gtf` file.

```
cellranger mkgtf \

genes.gtf \

genes_filtered.gtf \

-attribute=gene_biotype:protein_coding
```

d. Append `transgene.fa` to the provided `.fa` file
   i. create custom `.fa` file

```
touch transgene.fa
```

   ii. vim into the `.fa` file and add in the transgene information. Example (for *tdTomato* sequence) below.

```
>transgene 1443

CGGTACCGCCACCATGGTGAGTAAGGGCGAGGAAGTGATCAAAGAGTTCATGCGGTTTAAGGTGAGAATGGAAGGAAGCATGAAC
GGCCACGAGTTCGAAATTGAGGGAGAAGGAGAGGGACGGCCCTACGAGGGCACCCAGACAGCCAAGCTGAAAGTGACAAAGGGCG
GGCCTCTGCCATTCGCTTGGGACATCCTGAGCCCACAGTTTATGTACGGCTCCAAGGCCTATGTGAAACATCCAGCTGACATTCC
CGATTATAAGAAACTGAGCTTCCCCGAGGGGTTTAAGTGGGAAAGAGTGATGAACTTCGAGGACGGAGGCCTGGTGACTGTGACC
CAGGACAGCTCCCTGCAGGATGGGACCCTGATCTACAAGGTGAAAATGAGAGGGACAAATTTTCCCCCTGATGGACCTGTGATGC
AGAAGAAAACTATGGGATGGGAGGCCTCCACCGAAAGGCTGTATCCACGCGACGGGGTGCTGAAAGGAGAAATCCACCAGGCTCT
GAAGCTGAAAGATGGGGGACATTACCTGGTGGAGTTCAAGACAATCTACATGGCCAAGAAACCTGTGCAGCTGCCAGGCTACTAT
TACGTGGACACAAAACTGGATATCACTTCACACAACGAGGACTACACTATTGTGGAGCAGTATGAACGGAGCGAGGGGAGACACC
ATCTGTTCCTGGGCCATGGGACTGGAAGTACCGGCTCAGGGTCTAGTGGAACCGCCTCAAGCGAGGATAACAATATGGCTGTGAT
CAAAGAGTTCATGAGGTTTAAGGTGCGCATGGAGGGCAGCATGAATGGGCACGAATTTGAGATTGAAGGAGAGGGCGAAGGGAGG
CCTTACGAGGGCACACAGACTGCCAAGCTGAAAGTGACCAAGGGAGGACCACTGCCTTTCGCTTGGGATATCCTGTCTCCTCAGT
TTATGTACGGAAGTAAGGCCTATGTCAAGCATCCCGCTGACATTCCTGATTACAAGAAACTGTCTTTCCCAGAGGGCTTTAAGTG
GGAGAGAGTGATGAATTTTGAAGATGGAGGCCTGGTGACCGTGACACAGGACTCCTCTCTGCAGGATGGCACTCTGATCTACAAA
GTCAAAATGCGCGGCACCAATTTTCCACCCGATGGGCCCGTGATGCAGAAGAAAACAATGGGGTGGGAGGCCAGCACTGAACGGC
TGTATCCTAGAGACGGAGTGCTGAAGGGCGAAATCCACCAGGCCCTGAAGCTGAAAGACGGCGGCCACTACCTGGTGGAGTTCAA
AACCATCTACATGGCCAAGAAACCAGTGCAGCTGCCCGGCTATTACTATGTGGACACCAAGCTGGATATCACATCCCACAATGAA
GACTACACCATTGTGGAACAGTATGAGAGGTCTGAAGGACGCCACCATCTGTTTCTGTACGGCATGGATGAGCTGTATAAGTA
```

   iii. Append the custom `transgene.fa` file to the end of the provided `.fa` file.

```
cat transgene.fa >> genes.fa
```

e. Make the custom reference genome using the newly made `genes.fa` and `genes.gtf` files. Example code below:

```
cellranger mkref \

-genome=mm10_custom_ref \

-fasta=path_to_fa_file/genes.fa \

-genes=path_to_gtf_file/genes.gtf
```

f. Run *cellranger count* to generate counts for each gene in a cell count table that will be saved in a `.h5` file. Read more about this function here. We ran our parameters as follows:

```
cellranger count \

-id=sample_id \

-fastqs=path_to_fastqs \
```

```
-sample=sample_name_from_sequencer \
-include-introns \
-transcriptome=mm10_custom_ref
```

g. If combining samples, refer to instructions here. Example code below:

```
touch samples_to_aggregate.csv
```

```
vim samples_to_aggregate.csv
```

vim into the .csv file and add the sample information.

```
sample_id,molecule_h5
sample_01,sample_01_molecule_info_directory/molecule_info.h5
sample_02,sample_02_molecule_info_directory/molecule_info.h5
sample_03,sample_03_molecule_info_directory/molecule_info.h5
cellranger aggr -id=custom_name -csv=samples_to_aggregate.csv
```

h. Resulting count matrix will end in `.h5` file extension.
   i. example: `filtered_feature_matrix.h5`

Data analysis

Timing: 1–3 d

This section describes the procedures for clustering neurons, identifying markers, and overlaying viral barcode expression on clusters to identify projection neurons.

Single-cell sequencing analysis is commonly performed using *ScanPy* (*Python* package) or *Seurat* (*R* package). The below protocol describes steps using Python and *ScanPy* that were used in Cheung et al., (2021) (code). For clarity, our protocol references function names in *ScanPy* version 1.7.2, but please note that function names may change per authors and maintainers of the *ScanPy* library in future versions. Analogous steps can be performed using *R* and *Seurat*.

32. Hardware: We recommend a computer with at least 16 CPU cores, 64 GB RAM, and 1 TB of disk storage. The speed of some algorithms may benefit from additional CPU cores and it may be beneficial to use an on-demand cloud computing service to dynamically scale computing requirements since some (but not all) algorithms benefit from multi-core CPU acceleration.
33. Operating System:
    a. Install a Debian-based Linux distribution such as Ubuntu or Centos. We use Ubuntu in our examples.
    b. Using the package manager on the Linux distribution, install tools needed to configure the python environment, including *wget, sh, bash.*
34. Software:
    a. Download a python package manager such as *pip* or *conda.* We primarily used *conda* for package management and installations. Examples using *conda* below:

```
wget https://repo.continuum.io/miniconda/Miniconda3-latest-Linux-x86_64.sh -O ~/miniconda.sh
bash ~/miniconda.sh -b -p $HOME/miniconda
```

b. Create a conda environment <*env_name*> and then add bioconda and conda-forge channels. Then install packages. The specific packages shown below are used for the VECTORseq project. Specific versions of each package used can be found in the VECTORseq project code under the requirements.txt file.

```
conda config –add channels bioconda

conda config –add channels conda-forge

conda create –name <env_name> jupyter ipykernel nb_conda anndata==0.7.5 scanpy==1.7.2 leidenalg pysam pynndescent pandas==1.2.3 numpy scipy pytz matplotlib tqdm black flake8 scikit-learn pyarrow fastparquet snappy seaborn
```

35. Analysis: The following data pipeline is used for data cleaning and generating optimal clusters with biological plausibility for both inhibitory and excitatory neuronal datasets described in Cheung et al., (2021).
    a. Reformat: Convert data to *AnnData* format.
        i. Load the cell count table in the .h5 and convert it to the *AnnData* data format used by *ScanPy* using the function *scanpy.read_10x_h5*. This generates an in-memory *adata* object upon which downstream ScanPy functions can be applied.
        ii. In the VECTORseq project code, this step corresponds to the "reformat" pipeline stage.
    b. Distribution Plots: Assess sequencing data quality.
        i. Compute quality control metrics using the function *scanpy.pp.calculate_qc_metrics*.
        ii. Generate scatter plots and violin plots for number of genes, number of counts, and percent mitochondrial genes. These plots can reveal any contamination of single cell data with dead cells, doublets, multiplets, etc.
        iii. The percent mitochondrial genes cutoff we used was 5% for brain tissue.
        iv. General rule of thumb to plot the distribution across counts/cell and genes/cell.

    Note: For counts/cell, if there is a bimodal distribution, the initial filtration criteria is usually made on the value after the end of the first peak and at the beginning of the second one.

        v. In the VECTORseq project code, this step corresponds to the "distribution_plots" pipeline stage.
    c. Filter: Apply filtering cutoffs to remove dead cells, doublets, multiplets, and any unwanted artifacts created as a consequence of the single cell sequencing process that do not reflect biological phenomena .
        i. Apply count cutoffs using the function *scanpy.pp.filter_cells*.
        ii. You may also choose to selectively filter mRNA expression from chromosomal, ribosomal, and mitochondrial genes by manually subsetting *adata.X* and excluding a blacklist of known genes.
        iii. In the VECTORseq project code, this step corresponds to the "filter" pipeline stage.
    d. Normalize: Normalize counts and convert viral transgenes from expression data to a metadata label so that further analysis with *Scanpy* functions only operates upon endogenous genes.
        i. Log-normalize total counts (number of times gene detected) per cell using the functions *scanpy.pp.normalize_total* and *scanpy.pp.log1p*. This baseline count normalization is necessary for comparisons between cells. Apply any additional transforms as desired such as log transform, term frequency-inverse document frequency (TFIDF), depending on your application. TFIDF was used in Cheung et al., (2021) to highlight unique genes within each cell, including the unique viral transgenes that had been previously introduced.
        ii. Importantly, viral transgene expression should be removed from the cell count table and converted into a label since downstream steps are intended to be applied only to

endogenous gene expression. In *ScanPy* and *AnnData*, this can be accomplished by moving the viral transgenes and their corresponding counts from *adata.X* to *adata.var* which stores metadata corresponding to each cell. All further *ScanPy* methods will be applied to adata.X.

iii. Once adata.X contains only endogenous genes, several techniques are employed to reduce the number of genes to include in further analysis. TFIDF and log-normalization are repeated on only endogenous genes. Highly variable genes are computed using the function scanpy.pp.highly_variable_genes, both before and after TFIDF. Some genes may not be highly variable within a dataset but are useful for determination of cluster identity. To keep them in the dataset, we *a priori* whitelisted specific genes of interest. The final genes to be included in analysis are the set of genes in the whitelist combined with the union of highly variable genes before and after the TFIDF transform.

iv. Scale each gene to unit variance and clip outlier values using the function scanpy.pp.scale.

v. In the VECTORseq project code, these steps correspond to the "normalize" pipeline stage.

e. Cluster: Compute neighborhood graph and Leiden clusters.

i. Compute top *n* principal components of *adata.X* using *scanpy.pp.pca*, and then use *scanpy.pp*.neighbors to compute a neighborhood graph of observations using the top principal components. We selected an n of 50.

ii. Use the Leiden algorithm (Traag et al., 2019) with scanpy.tl.leiden to generate clusters based on the neighborhood graph. Various results can be obtained depending on how many n principal components are selected, the n_neighbors used to generate the neighborhood graph, and the leiden_resolution used for Leiden clustering. Using n = 50, we ran a grid search for n_neighbors and leiden_resolution. The value of n_neighbors is swept from 5 to 105 with increments of 5. The value of leiden_resolution is swept from 0.05 to 1.25 with increments of 0.05.

iii. In the VECTORseq project code, this step corresponds to the "cluster" pipeline stage.

f. Cluster Metrics:

i. Compute various internal cluster validation metrics for each combination of n_neighbors and leiden_resolution used in the grid search. Internal cluster validation metrics include within-cluster sum of squared error, within-cluster variance, Davies-Bouldin Index, Average Silhouette Score, Calinski-Harabasz Index (Pseudo-F statistic), and Xie-Beni Index (Caliński and Harabasz, 1974; Davies and Bouldin, 1979; Rousseeuw, 1987; Singh et al., 2017).

ii. Visualize internal cluster validation metrics on heatmaps and 3D surface plots. Local minima and maxima on these plots correspond to more optimal hyperparameters.

Note: In general better separation of clusters is achieved by minimizing within-cluster sum of squared error, within-cluster variance, Davies-Bouldin Index, Xie-Beni Index, and by maximizing Calinski-Harabasz Index and Average Silhouette Score.

iii. Multiple local minima/maxima may exist for each of the internal cluster validation metrics. These local optima suggest good candidate options for hyperparameters *n_neighbors* and *leiden*_resolution.

Note: Final selection of these hyperparameters ultimately requires inspection of cluster gene expressions and correlation with biological plausibility.

iv. In the VECTORseq project code, this step corresponds to the "cluster_metrics" pipeline stage.

g. Create UMAP: Generate Uniform Manifold Approximation and Projection (UMAP) visualizations.

i. Use UMAP to remap the high-dimensional gene expression manifold to a 2D representation with *scanpy.tl.umap*, then use *scanpy.pl.umap* to create a scatter plot. Color code

cluster identities generated from Leiden clustering onto the UMAP plot. A unique UMAP visualization is generated for every combination of *n_neighbors* and *leiden_resolution* used in the grid search.

ii. Using a combination of internal cluster validation metrics and inspection of UMAP visualization along with the biological plausibility of known genes from the whitelist of genes included during the Normalize step, optimal clustering schemes with specific *n_neighbors* and *leiden_resolution* can be selected for further downstream analysis.

Note: Selection of specific *n_neighbors* and *leiden_resolution* will vary between datasets. Cross-examining several different combinations via exploratory data analysis will be the most useful in determining which parameters to choose.

iii. In the VECTORseq project code, this step corresponds to the "create_umap" pipeline stage.

h. Expression Plots: Generate gene expression plots.

i. After an optimal combination of *n_neighbors* and *leiden_resolution* is chosen, use the cluster identities to generate a dendrogram plot using *scanpy.tl.dendrogram* and *scanpy.pl.dendrogram*.

ii. Relative gene expressions can be visualized with heatmaps using *scanpy.pl.heatmap*, matrix plots using *scanpy.pl.matrixplot*, and dot plots using *scanpy.pl.dotplot*. When calling these functions, group by the leiden cluster identities and include the computed dendrogram to help visualize cluster relationships.

iii. Most highly expressed genes within each cluster group can be selectively visualized. Genes within each cluster can be ranked using *scanpy.tl.rank_genes_groups*, then visualized as a heatmap using *scanpy.pl.rank_genes_groups_heatmap*, matrix plots using *scanpy.pl.rank_genes_groups_matrixplot*, and dot plots using *scanpy.pl.rank_genes_groups_dotplot*. When calling these functions, group by the Leiden cluster identities and include the computed dendrogram to help visualize relationships. You will also need to specify how many top ranked genes in each cluster you desire to visualize on the plots.

iv. At this stage, expression of viral transgenes can be overlaid to identify the clusters corresponding to different projection populations.

Note: These annotations were stored in *adata.var* during our normalization step. Viral overlays can be performed *ScanPy*'s built-in plotting functions by calling the column name used when storing the annotation in *adata.var*.

v. In the VECTORseq project code, this step corresponds to the "expression_plots" pipeline stage.

i. **Subset:** Subset data based on selected clusters to further explore expression data.

i. This is an optional step which can be recursively performed to target specific subset of cells based on cluster membership. For example, in Cheung et al., (2021), we extracted only excitatory neuron clusters based on gene expression profile, then performed further clustering and expression analysis on that subset by repeating the above steps Cluster, Cluster Metrics, Create UMAP, and Expression Plots. The same was done for inhibitory neuron clusters.

ii. In the VECTORseq project code, this step corresponds to the "subset" pipeline stage.

EXPECTED OUTCOMES

The expected outcome of this protocol is transcriptional delineation of the neuronal cell types within a structure with different projection types transcriptionally marked via transgene expression (Figure 5). In a successful experiment, viral transgenes will be selectively enriched in one or a few cell

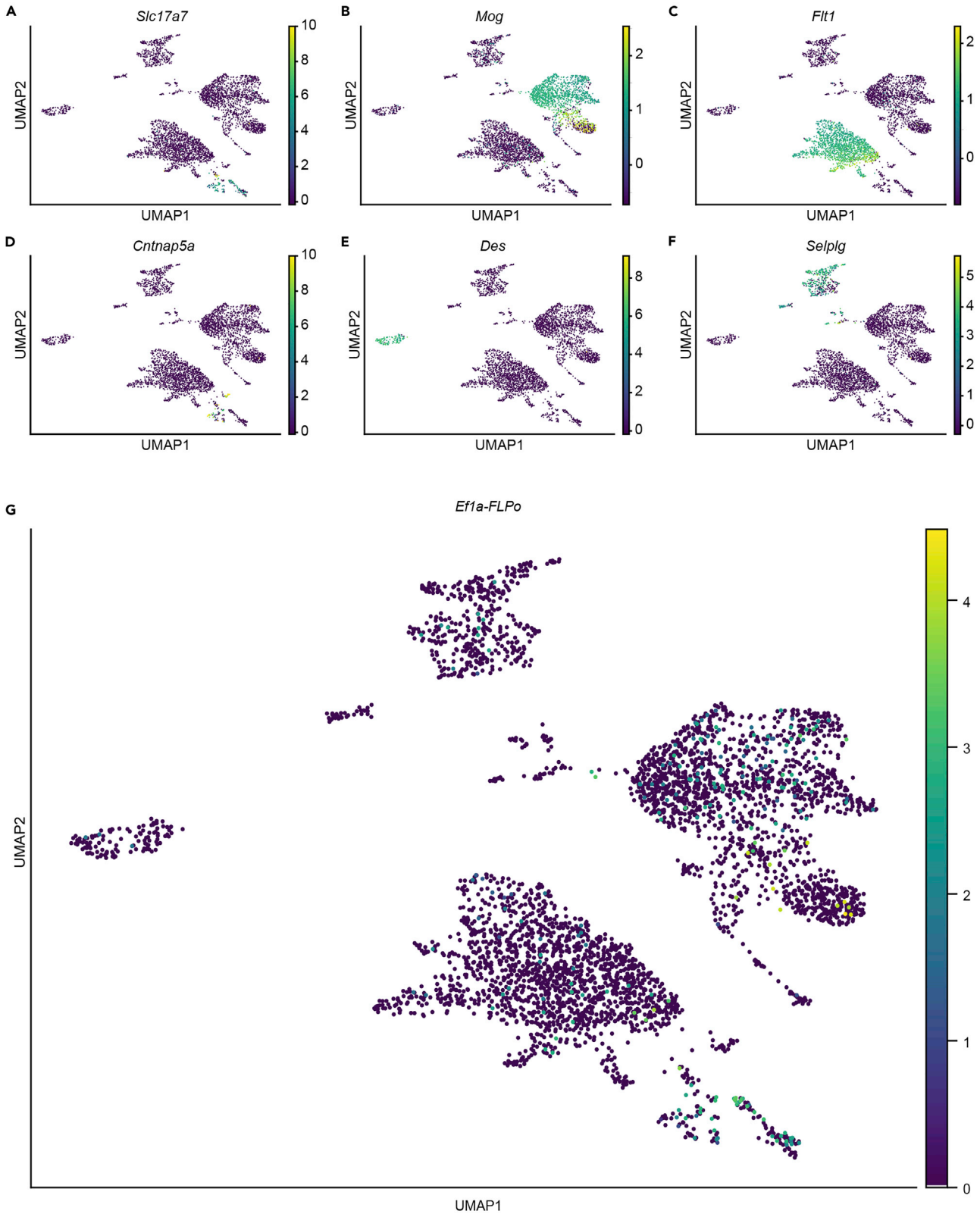

A
Slc17a7
B
Mog
C
Flt1
D
Cntnap5a
E
Des
F
Selplg
G
Ef1a-FLPo
UMAP1
UMAP2

Figure 5. Example of expected outcomes

(A–F) UMAP plots for V1 VECTORseq dataset showing distributions of markers for major cell types (excitatory neurons, oligodendrocytes, endothelia, inhibitory neurons, mural cells, and microglia, respectively).

(G) UMAP plot with expression of retrograde viral transgene (FLPo) overlaid. Note marked enrichment in cluster that expresses excitatory marker (Slc17a7), as expected.

types that project to the target structure; ubiquitous expression, if observed, is likely due to ambient mRNA contamination (see limitations).

LIMITATIONS

The largest concern in any VECTORseq experiment is viral tropism. It is essential to confirm that any virus and batch to be used in sequencing experiments infects the projection population of interest. Likewise, mixing a locally infecting virus encoding a fluorophore with the retrograde viruses allows validation of the site of infection in individual animals during tissue slicing, which is also useful for identifying mice within a cohort with injection failures or mistargeting that should be excluded from the pooled sequencing reaction. In addition, we found that spillover from viral expression within a projection target near the source structure yielded abundant ambient mRNA in all the sequenced populations. Therefore, when investigating projections to structures near the source structure, it may be necessary to ensure that dissections exclude the injection site or to use a retrograde virus such as HSV, which is reported to express stably in retrogradely infected neurons but only transiently in neurons at the injection site (Fenno et al., 2014). Similarly, when different cells within a source structure project to neighboring targets, it is important to ensure that viral injections into the target structure are focal, e.g., by limiting injection volume, in order to avoid spillover. Another concern is that stereotaxic targeting may be impaired in certain models of pathological conditions. Therefore, it is critical to validate the anatomy of the specific strain, age, and model under study. In addition, adjustments to the code and troubleshooting may be necessary for other experiments and brain areas.

A potential concern is that viruses may alter endogenous physiology and gene expression of infected cells. To this end, it is helpful to use viruses known to have minimal effects on cellular physiology such as AAV and HSV. In addition, one can take advantage of the fact that not every cell in a given population will be infected and compare gene expression between infected and uninfected cells within a given cluster; any changes in gene expression in infected cells, particulary induction of inflammatory or other immune genes, may indicate cellular perturbations by viral infection. Note that in our initial study, these analyses found that HSV and AAV did not alter endogenous gene expression in any of the cell types we analyzed, but similar analyses are worthwhile for each dataset. As an additional control for the effects of viral expression, one could sequence an uninfected cohort to compare the abundance of different cell clusters and gene expression within clusters.

Finally, although NeuN labeling can helpfully enrich for most neuronal nuclei, it is important to realize that not every neuronal cell type expresses NeuN and thus this enrichment approach could lose certain cell types.

TROUBLESHOOTING

Problem 1

Lack of viral infection or spillover from injection site (stereotaxic injections, step 4).

Potential solution

A combination of test retrograde and anterograde infections followed by histology to ensure that the viruses infect the cell types of interest and the lack of spillover from the injection site.

Problem 2
Differences in neuroanatomy across strains, ages, and/or disease models.

Potential solution
Confirm stereotaxic targeting of the structures of interest in the relevant model (stereotaxic injections, step 4).

Problem 3
Possibility that viral infections cause cytotoxicity and/or altered gene expression.

Potential solution
Perform within-cluster comparisons of gene expression in virally labeled and unlabeled cells or even, when available, to compare clusters with those obtained from sequencing of an uninfected cohort (data analysis, step 35hiv).

Problem 4
The most costly and time-intensive steps are preparing, sequencing, and analyzing the 10x library.

Potential solution
We recommend practicing the cell/nuclei isolation steps on the tissue of interest in uninfected animals before attempting to generate libraries and sequencing a VECTORseq cohort (single-cell isolation, single-nuclei isolation).

Problem 5
Potential problems with library preparation.

Potential solution
It is good practice to sequence each library shallowly and inexpensively at first to identify any issues with the library preparation before doing a deeper and more expensive sequencing run for final analyses (Library preparation).

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Evan Feinberg (evan.feinberg@ucsf.edu).

Materials availability
This study did not generate new unique reagents. Mice and all reagents used in this study are commercially available as indicated in the key resources table.

Data and code availability
- The raw and processed single-cell sequencing data are deposited at https://ucsf.app.box.com/v/vectorseq-data and can be found on NCBI GEO (GSE189907).
- All code for analysis is publicly available at https://github.com/vic-cheung/vectorseq and https://doi.org/10.5281/zenodo.5703724.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS
This work was supported by departmental funds and grants from the E. M. Ziegler Foundation for the Blind, Sandler Foundation, Klingenstein-Simons Fellowship Award in Neuroscience, Brain and Behavior Research Foundation (NARSAD Young Investigator Awards 25337 and 27320), Whitehall Foundation, Simons Foundation (SFARI 574347), and US National Institutes of Health (DP2

MH119426 and R01 NS109060) to E.H.F. We thank C. Cheung for help with software development and code refactorization. We thank J. McGuire and M. Bernardi at the Gladstone genomics core for 10X library preparation and sequencing and staff at the UCSF Center for Advanced Technology (CAT) for support with RNA sequencing. Flow cytometry was performed by the Gladstone flow cytometry core, which is supported by NIH S10 RR028962, James B. Pendleton Charitable Trust, and NIH P30 AI027763.

AUTHOR CONTRIBUTIONS

V.C.: Methodology, Software, Validation, Formal Analysis, Investigation, Resources, Data Curation, Writing—original draft, Writing—Review & Editing, Visualization, Project Administration; P.C.: Software, Validation, Formal analysis, Writing—Review & Editing, Visualization; E.H.F.: Conceptualization, Methodology, Resources, Writing—original draft, Writing—Review & Editing, Visualization, Project Administration, Funding Acquisition, Supervision

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Caliński, T., and Harabasz, J. (1974). A dendrite method for cluster analysis. Comm. Stats. - Theor. Methods *3*, 1–27.

Cheung, V., Chung, P., Bjorni, M., Shvareva, V.A., Lopez, Y.C., and Feinberg, E.H. (2021). Virally Encoded Connectivity Transgenic Overlay RNA sequencing (VECTORseq) defines projection neurons involved in sensorimotor integration. Cell Rep. *37*, 110131.

Davies, D.L., and Bouldin, D.W. (1979). A cluster separation measure. IEEE Trans. Pattern Anal. Mach. Intell. *1*, 224–227.

Krishnaswami, S.R., Grindberg, R.V., Novotny, M., Venepally, P., Lacar, B., Bhutani, K., Linker, S.B., Pham, S., Erwin, J.A., Miller, J.A., et al. (2016). Using single nuclei for RNA-seq to capture the transcriptome of postmortem neurons. Nat. Protoc. *11*, 499–524.

Martin, C., Abdul, A., Vanderburg, C., Nadaf, N., Feirrera, A., and Macosko, E.. Frozen Tissue Nuclei Extraction (for 10xV3 snSEQ). **protocols.io**. https://dx.doi.org/10.17504/protocols.io.bck6iuze.

Rousseeuw, P.J. (1987). Silhouettes: a graphical aid to the interpretation and validation of cluster analysis,. J. Comput. Appl. Maths. *20*, 53–65. ISSN 0377-0427.

Singh, M., Bhattacharjee, R., Sharma, N., and Verma, A. (2017). An improved xie-beni index for cluster validity measure. In 2017 Fourth International Conference on Image Information Processing (ICIIP), pp. 1–5.

Traag, V.A., Waltman, L., and van Eck, N.J. (2019). From Louvain to Leiden: guaranteeing well-connected communities. Sci. Rep. *91*, 5233–5312.

Wolf, F.A., Angerer, P., and Theis, F.J. (2018). SCANPY: large-scale single-cell gene expression data analysis. Genome Biol. *19*, 15.

Wolock, S.L., Lopez, R., and Klein, A.M. (2019). Scrublet: computational identification of cell doublets in single-cell transcriptomic data. Cell Syst. *8*, 281–291.e9.

Zheng, G.X.Y., Terry, J.M., Belgrader, P., Ryvkin, P., Bent, Z.W., Wilson, R., Ziraldo, S.B., Wheeler, T.D., McDermott, G.P., Zhu, J., et al. (2017). Massively parallel digital transcriptional profiling of single cells. Nat. Commun. *8*, 14049–14112.