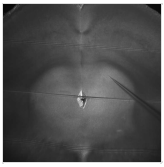

Aug 11, 2021

Visually guided aspiration of fluorescently labelled single neurons from acute midbrain slices followed by Smart-seq2

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DISCLAIMER

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We would like to thank Petr Znamenskyi, Yoh Isogai, Panagiota Iordanidou, Mathew Edwards, and Molly Strom for their help and advice prior to and during the development of this protocol. We would also like to thank Robb Barrett from the Fabrications Lab at SWC for designing and building the box to store the aspiration pipettes.

ABSTRACT

To link the expression of ion channels, receptors, and molecular effectors to specific brain areas and their subdivisions, it is necessary to perform single-cell RNA-sequencing while preserving the anatomical origin of each neuron. Here we describe a protocol to perform visually guided aspiration of fluorescently labelled single neurons from acute midbrain slices of transgenic mice, followed by Smart-seq2 and deep sequencing. This protocol enables researchers to obtain detailed transcriptomic profiles from any cell-type labelled using transgenic lines - and potentially other methods like viral injections - located in a brain area of interest.

DOI

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PROTOCOL CITATION

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KEYWORDS

Mouse, Acute Brain Slices, Midbrain, Single-cell RNA sequencing, Single-cell isolation, Smart-seq2, Neuroscience, Transcriptomics

IMAGE ATTRIBUTION

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GUIDELINES

A typical experiment consists on preparing the slices in the morning, do the incubation steps during lunch, and start collecting cells right after lunch. Once collected, store the isolated cells at -80°C until there are enough to process with the Smart-seq2 protocol. Once processed and checked for quality, keep the samples at -20°C until there are enough to send for sequencing.

MATERIALS TEXT

General reagents and consumables:

- RNaseZap (Ambion or Invitrogen, Cat. No. AM9780, £50.55).
- DNA-OFF (Takara, Cat. No. 9036, £65.70).
- Mouse Brain Total RNA to optimise the protocol (Takara, Cat. No. 636601, £225).
- PCR tubes (0.2 mL, Alpha Laboratories, Cat. No. LW2570, £42).
- Freezer storage box for 0.2 mL PCR tubes (Bel Art Products, Cat. No. 15427710, £27.95).
- Cryo-Mini-Dot labels for 0.2 mL PCR tubes (Tag Scientific, Cat. No. WRPLT-7A, £38.10).

Solutions and tools to prepare acute midbrain slices for single-cell aspiration:

- See https://dx.doi.org/10.17504/protocols.io.bukhnut6 for a full list of materials and a detailed step-by-step protocol.
- Unless you plan to obtain whole-cell recordings from the target cell before aspirating it, there is no need to use normal internal solution and blow potassium to all the cells while you approach the target cell. Instead, use recording ACSF to fill the pipettes used to aspirate cells. Importantly, add 2 U/µL of recombinant RNase inhibitor to prevent RNA degradation (e.g. add 15 µL of RNAseOUT to 285 µL of recording ACSF).

Smart-seq2 primers:

Name	Sequence (5'-3')	Use	Source
Oligo-dT primer (pi5)	AAGCAGTGGTATCAACGCAGAGTACT30CG	Anneals to RNAs containing a poly(A) tail	Sigma
Oligo-dT primer (pi6)	AAGCAGTGGTATCAACGCAGAGTACT30AG	Anneals to RNAs containing a poly(A) tail	Sigma
LNA-modified TSO	AAGCAGTGGTATCAACGCAGAGTACATrGrG+G	Template Switching Oligonucleotide	Qiagen
ISPCR	AAGCAGTGGTATCAACGCAGAGT	PCR preamplification primer	Sigma

List of oligos and primers used.

Smart-seq2 lysis buffer:

- Recombinant RNase inhibitor, to prevent RNA degradation (Clontech, Cat. No. 2313A, £86.40).
- Triton X-100, detergent to dissolve the cell membrane to retrieve its RNA contents (0.2%, Sigma, Cat. No. T9284, £44.54).
- ERCC RNA spike-in Mix 1, RNA transcripts of known sequence and quantity to act as positive controls (1:500,000 dilution, Thermo Fisher, Cat. No. 4456740, £974).
- dNTP mix, for DNA strand building (Thermo Fisher, Cat. No. R0192, £58.22).
- oligo-dT_{30}VN primer (Sigma, HPLC purified).

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**From Picelli et al. Nature Methods 2013: the average length of the preamplified cDNA increased when adding dNTPs before the RNA denaturation rather than in the reverse transcription master mix, presumably through mechanisms that stabilize the hybridization of RNA to the oligo-dT primer.*

From Picelli et al. Nature Protocols 2014: the oligo-dT primer anneals to all the RNAs containing a poly(A) tail. The 3' end of this oligonucleotide contains 'VN', where 'N' is any base and 'V' is either A, C or G. The two terminal nucleotides are necessary for anchoring the oligonucleotide to the beginning of the polyA tail and to avoid unnecessary amplification of a long stretch of adenosines. Dissolve the oligonucleotide in TE buffer, to a final concentration of 100 µM. Store this oligo at −20°C for 6 months. **TE buffer to solubilize DNA/RNA and prevent degradation: Tris (pH buffer) and EDTA (chelates cations eg. Mg2+).*

Smart-seq2 cDNA synthesis - reverse transcription master mix:

- Product of the lysis buffer reaction (see above).
- Recombinant RNase inhibitor, to prevent RNA degradation (Clontech, Cat. No. 2313A, £86.40).
- SuperScript II reverse transcriptase, to transcribe cDNA from RNA (Invitrogen, Cat. No. 18064-014, £156.33). *The reverse transcriptase (M-MLV) transcribes cDNA from RNA and allows the recovery of full-length cDNAs, owing to the preference of M-MLV reverse transcriptase for full-length over truncated cDNAs as a substrate for its terminal transferase activity.*
- SuperScript II first-strand buffer (5x, Invitrogen, Cat. No. 18064-014).
- DTT (100 mM, Invitrogen, Cat. No. 18064-014). *Stabilises enzymes and breaks down disulfide bonds. Also works as a reducing agent, preventing some DNA dimerization.*
- Betaine (5 M, Sigma, Cat. No. B0300-1VL, £12.98). *Reduces formation of secondary structure in GC rich regions.*
- Magnesium chloride (1 M, Sigma, Cat. No. M8266, £11.97). *The presence of the methyl group donor betaine in combination with higher MgCl2 concentrations (9-12 mM) significantly increases yield.*
- Nuclease-free water (Qiagen, Cat. No. 10977035, £9.49).
- Template switching oligonucleotide (100 µM, TSO, Qiagen, Cat. No. 339412, £348.7).

**From Picelli et al. Nature Methods 2013: exchanging only a single guanylate for a locked nucleic acid (LNA) guanylate at the TSO 3' end (rGrG+G) led to a two-fold increase in cDNA yield relative to that obtained with the SMARTer IIA oligo. This is likely a consequence of the increased thermal stability of LNA:DNA base pairs (1-8 °C per LNA monomer).*

**From Picelli et al. Nature Protocols 2014: at the 5' end, this TSO carries a common primer sequence, whereas, at the 3' end, there are two riboguanosines (rG) and one LNA-modified guanosine (+G) to facilitate template switching. Dissolve TSO in TE buffer. TSO can be stored in 100 µM aliquots at −80°C for 6 months. Avoid repeated freeze-thaw cycles.*

Smart-seq2 PCR preamplification mix:

- Product of the reverse transcription reaction (see above).
- KAPA HiFi Hotstart ReadyMix (2x, KAPA Biosystems, Cat. No. KK2601, £104.26).
- ISPCR primer (10 µM, Sigma).
- Nuclease-free water (Qiagen, Cat. No. 10977035).

**From Picelli et al. Nature Methods 2013: we noted that KAPA HiFi Hot Start (KAPA) DNA Polymerase efficiently amplified first-strand cDNA directly after reverse transcription, with no need for prior bead purification. Importantly, libraries preamplified without bead purification had no reduction in yield, but their average cDNA length was 450 nt greater. Preamplification using KAPA improved GC tolerance and read coverage across transcripts, but they also suggest that comparing data generated using different amplifications procedures could be complicated.*

**From Picelli et al. Nature Protocols 2014: the ISPCR oligo acts as PCR primer in the amplification step after RT. Dissolve the oligonucleotide in TE buffer to a final concentration of 100 µM. This oligo can be stored at−20 °C for 6 months.*

Smart-seq2 cDNA purification:

- Ampure XP beads (Beckman Coulter, Cat. No. A63880, £200.15).
- Magnetic stand (Invitrogen, Cat. No. AM10027, £324.44).
- Ethanol (VWR, Cat. No. 20821.330).
- Elution buffer solution (Qiagen, Cat. No. 19086, £27.19).

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Quality checks of the cDNA library:

- NanoDrop 2000 spectrophotometer (Thermo Scientific).
- Qubit dsDNA High-Sensitivity Assay Kit (Invitrogen, Cat. No. Q32851, £56.16) in a Qubit 2.0 fluorometer (Life Technologies).
- Agilent High-Sensitivity DNA kit (Agilent Technologies, Cat. No. 5067-4626, £313.49) run in an Agilent 2100 Bioanalyzer (Agilent Technologies).

Tagmentation, library pooling, and sequencing:

These steps were done externally by the Barts London Genome Centre at the Blizard Institute (Barts and The London School of Medicine and Dentistry, Queen Mary University of London). Libraries were prepared using the Illumina Nextera XT Sample Preparation Kit (Illumina Inc.) with an input of 150 pg of cDNA per sample. Resulting libraries were checked for average fragment size using the Agilent D1000 ScreenTape (Agilent Technologies) and were quantified using the Qubit dsDNA High-Sensitivity Assay Kit. Equimolar quantities of each sample library were pooled together and 75 bp paired-end reads were generated for each library using the Illumina NextSeq 500 High-output sequencing kit, with a target sequencing depth of ~4.16 M reads per sample.

Other equipment:

- P-1000 Micropipette Puller to obtain 1-2 MΩ pipettes (Sutter Instruments, ~£7000).
- Borosilicate standard wall with filament capillary glass, 1.5 mm OD, 0.86 mm ID, 100 mm L (Cat. No. 300057 - GC150F-10, Pack of 225 capillary glass, Harvard Apparatus via MultiChannel Systems, €46 each pack).
- Micropipette 2-20 µL to backfill glass recording electrodes (Cat. no:. 17014412, Mettler Toledo, Rainin) and Microloader Tips (Cat. No. 5242956003, Eppendorf).
- Oven to bake and sterilize glassware and glass capillaries (Binder Oven E28, Wolf Labs, £544).
- Thermal cycler (T100, Bio-Rad).
- Mini vortex mixer (VWR).
- Filter tips dedicated for RNA-work: 10, 20, and 200 µl (Starlabs, Cat. No. S1121-3810, S1123-1810, S1120-8810)
- Qubit assay tubes (Invitrogen, cat. no. Q32856, £43.74).
- 1 mm stage microruler with 10 µm divisions to calibrate microscope images (Thorlabs, Cat. No. R1L3S2P).

SAFETY WARNINGS

This protocol involves animal work and potential exposure to allergens, always wear appropriate personal protective equipment (PPE) and follow all the protocols in place at your home institution.

DISCLAIMER:

This work was funded by a Wellcome Senior Research Fellowship (214352/Z/18/Z) and a Sainsbury Wellcome Centre Core Grant from the Gatsby Charitable Foundation and Wellcome (090843/F/09/Z) (T.B.), a UCL Wellcome 4-year PhD Programme in Neuroscience Fellowship (203729/Z/16/Z) (O.P.A.), and the SWC PhD Programme (S.F.O.).

We would like to thank Petr Znamenskyi, Yoh Isogai, Panagiota Iordanidou, Mathew Edwards, and Molly Strom for their help and advice prior to and during the development of this protocol. We would also like to thank Robb Barrett from the Fabrications Lab at SWC for designing and building the box to store the aspiration pipettes.

BEFORE STARTING

Ensure any animal work is carried out following the legislation and protocols in place at your home institution. Make sure you have all the materials in advance - some reagents can take a few days or weeks to arrive.

General considerations

1

This protocol has been adapted and modified from:

Citation: Oriol Pavon Arocas, Sarah F. Olesen, Tiago Branco (08/11/2021). Visually guided aspiration of fluorescently labelled single neurons from acute midbrain slices followed by Smart-seq2. https://dx.doi.org/10.17504/protocols.io.bhfxj3pn

- S. Picelli, O. Faridani, Å. Björklund, et al. Nature Protocols 2014. Full-length RNA-seq from single cells using Smart-seq2. DOI: https://doi.org/10.1038/nprot.2014.006
- Petr Znamenskiy - Benchling Protocol 2015 - Single cell RNAseq from acute cortical slices

Further details for the tools and steps related to brain extraction and preparation of acute midbrain slices can be found on Oriol Pavon Arocas, Tiago Branco (2021). Preparation of acute midbrain slices for patch-clamp recordings. [https://dx.doi.org/10.17504/protocols.io.bukhnut6]

2 General advice:

- Set aside a clean area for pre-PCR steps - ideally a UV-sterilized hood with laminar flow, with all the surfaces free from RNase, to prevent degradation of RNA, and from DNA, to prevent cross-contamination from previous samples.The hood must be used only for single-cell experiments up to (but excluding) the cDNA amplification step.
- Clean all surfaces and tools with RNaseZap and DNA-OFF to prevent RNA degradation and DNA contamination.
- Change gloves frequently.
- **Micropipette puller**: clean gloves and outer surfaces with RNaseZap before pulling the pipettes.
- **Throughput**: a typical experiment allows collection of ~24 cells, ~85% of which yield good cDNA libraries.
- **Freezing point**: freeze the lyzates (aspirated cell in lysis buffer) in dry ice immediately after aspiration, store them at -80°C for a few days, and thaw them right before starting the Smart-seq2 protocol.
- **Optimizing the protocol**: perform a test run of the protocol with mouse brain total RNA before trying it on aspirated cells. Always run no RNA negative controls too (e.g. empty pipette or pipette inserted into the slice but without aspirating a cell).
- **Lysis buffer**: prepare fresh on the day, it shouldn't be freeze-thawed.
- **Logistics**: in an ideal scenario a two-person team work in parallel - while one prepares the acute brain slices the other prepares the lysis buffer, so that it is fresh for the day.

Preparation of tools, instruments, and solutions for the day 4h

3 [Bake glass capillaries - previous day]

- Use standard-walled filament-containing borosilicate glass capillaries (Harvard Apparatus).
- On the day before the experiment, flame the edges of each capillary to smooth the glass. Carefully place all the flamed capillaries on a borosilicate glass beaker and cover them with aluminum foil to prevent dust from getting in. Bake them in an oven (Binder) at 200°C for 2 hours to sterilize them. Account for the time needed for the oven to reach the target temperature (e.g. the oven takes ~30 minutes to reach 200ºC).
- Make sure everything is ready for the experiment taking place on the next day, including the animal and the solutions to prepare acute midbrain slices.

4 [Prepare tools, instruments, and solutions for acute midbrain slices]

- To prepare acute midbrain slices, follow the steps described in Oriol Pavon Arocas, Tiago Branco (2021). Preparation of acute midbrain slices for patch-clamp recordings. [https://dx.doi.org/10.17504/protocols.io.bukhnut6] or any similar protocol that works for your brain region of interest.
- Have all the solutions and tools ready before proceeding to pull the pipettes. The optimal order would be: bake glass capillaries on the previous day, prepare tools and solutions on the day, pull pipettes, and finally prepare slices.

5 [Pull pipettes]

- Take the time to design and thoroughly test a program that reliably pulls pipettes with a 1-2 MΩ resistance. Smaller pipettes will make it harder to aspirate the target cells, whereas smaller ones may aspirate unwanted tissue from the surrounding area.
- Wear gloves and spray them with RNaseZap. Only then get the baked capillaries from the oven.
- Take the capillaries, the RNaseZap, and a box to keep the pipettes to where the puller is.
- Clean the surfaces, the parts of the puller you will touch, and the pipette storage box with RNaseZap. Put some tissue paper on the bench and place the box and the beaker with the baked capillaries on it.

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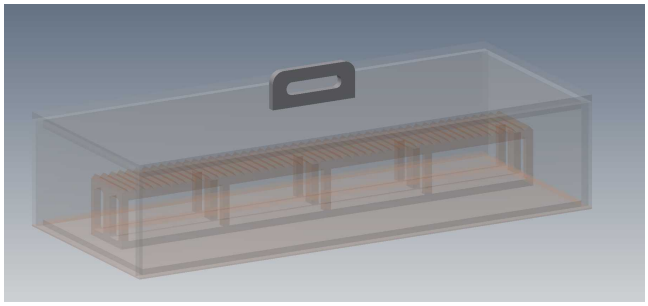
- Proceed to pull enough pipettes for the day: a typical experiment allows collection of ~24 cells, for which ~50 pipettes are usually enough (take into account that some will break and others will be lost during failed collection attempts).
- It takes ~50 minutes to pull 50 pipettes on the horizontal puller.



Example of a box with the capacity to store up to 60 pulled pipettes.
Clean with RNaseZap before use.

5.1 Finding the right program will take several rounds of testing. The Sutter Pipette Cookbook is a very useful guide, and keeping the following things in mind will make the process a bit easier:

- Enable the ThermoLock option, which preheats the Jaws to 70°C and reduces variability in successive rounds of pulling.
- Run a ramp test before starting the testing (and do it again if you replace the filament or a once stable program becomes unstable).
- Find the mid-point velocity.
- Once you have the mid-point velocity that results in the number of loops and resistance closer to your desired value, you can proceed to tweak the temperature.
- Pull several pipettes in a row with the same program and then test their resistance. Tweak the temperature parameter and iterate until you find a stable and reliable program.

As an example, the table below contains the program used on a P-1000 Sutter Puller to achieve 1-2 MΩ pipettes. The exact values may differ across machines and models and they will very likely need to be adjusted if the filament is replaced or a new ramp value is set.

Steps	Heat	Pull	Velocity	Delay	Pressure
7x	525	0	15	1	600

Example program to pull 1-2 MΩ pipettes on a P-1000 Sutter Puller.

6 [Prepare acute midbrain slices]

- Follow the steps described in Oriol Pavon Arocas, Tiago Branco (2021). Preparation of acute midbrain slices for patch-clamp recordings. [https://dx.doi.org/10.17504/protocols.io.bukhnut6] or any similar protocol that works for your brain region of interest.

7 [Prepare lysis buffer]

You can prepare the lysis buffer during the incubation period of the acute slices. However, in an ideal scenario a two-people team work in parallel: while one prepares the slices the other prepares the lysis buffer.

7.1 ⚠

Clean the hood with RNaseZap and DNA-OFF solutions before setting up the working plates. Spray

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pipettes and scissors (used to cut PCR tubes) with RNaseZap.

7.2 Calculate the volume you will need for the number of samples you want to collect. For one sample, you will need 4 µL of lysis buffer (see next step for exact volumes). Prepare enough lysis buffer for 2 extra samples, just to be safe and account for any volume lost during aliquoting.

- *[Done in advance] Dilute the oligo-dT$_{30}$VN primer to 10 µM by adding 10 µL of 100 µM oligo-dT primers and 90 µL of nuclease-free water to a tube and mix well. Aliquot and freeze.*
- *[Done in advance] Dilute and aliquot the ERCC Spike-ins to the desired dilution (e.g. 1:500,000).*
- Thaw reagents for Smart-seq2 lysis buffer.

7.3 For each sample, add the following to a 0.2 mL thin-walled PCR tube:

- 1.8 µL 0.2% Triton X-100.
- 0.1 µL recombinant RNase inhibitor.
- 0.1 µL ERCC RNA spike-in Mix 1 (1:500,000 dilution).
- 1 µL oligo-dT$_{30}$VN primer (10 µM).
- 1 µL dNTP mix (10 µM).

Keep the tubes with the lysis buffer on ice until and during cell collection.

As an example, to aspirate 24 cells prepare enough lysis buffer for 30 samples. This will usually yield ~28 PCR tubes with 4 µL of lysis buffer each (some volume gets lost when pipetting). This ensures you have enough tubes for 24 samples, 1 negative control, and 3 extra just in case accidents happen or collection goes wrong.

Calculate the final volumes in advance and add everything together before aliquoting to the small PCR tubes. Add 54 µL 0.2% Triton X-100, 3 µL recombinant RNase inhibitor, 3 µL ERCC RNA spike in Mix 1 (1:500,000 dilution), 30 µL oligo-dT$_{30}$VN primer (10 µM), and 30 µL dNTP mix (10 µM) for a total volume of 120 µL.

Isolation of single-cells from acute midbrain slices 6h

8 [Collection of single-cells and Single-cell lysis]

Once the incubation period is over, the slices are ready and you can begin cell collection. With this visually guided aspiration approach, sample collection is quite slow. On a typical experiment, you can proceed to manually aspirate cells for up to 5-7 hours from the moment the slices are ready, and a total of 24 cells are usually collected. Take care to isolate cells in equal proportions from each hemisphere and brain area subdivision, following a sequence pseudo-randomised for each animal. A negative control containing only the 4 µL of lysis buffer should also be included for each batch of 24 cells to assess DNA contamination during the processing steps. To assess the amount of ambient mRNA added by the isolation method, negative controls can be obtained by inserting the glass pipette in the slice tissue without aspirating any cell. In some instances, whole-cell patch-clamp recordings can be obtained from the target neuron prior to aspiration.

8.1 Before commencing sample collection, prepare an aliquot of the solution that will be used to fill the pipettes before aspirating the cells. Unless you aim to perform whole-cell recordings before aspiration (i.e. patch-seq), use recording ACSF containing 2 U/µL recombinant RNase inhibitor, prepared by mixing 15 µL of RNase inhibitor and 285 µL of recording ACSF. Keep on ice for the length of the experiment.

- Transfer a slice to a submersion type recording chamber continuously perfused with recording

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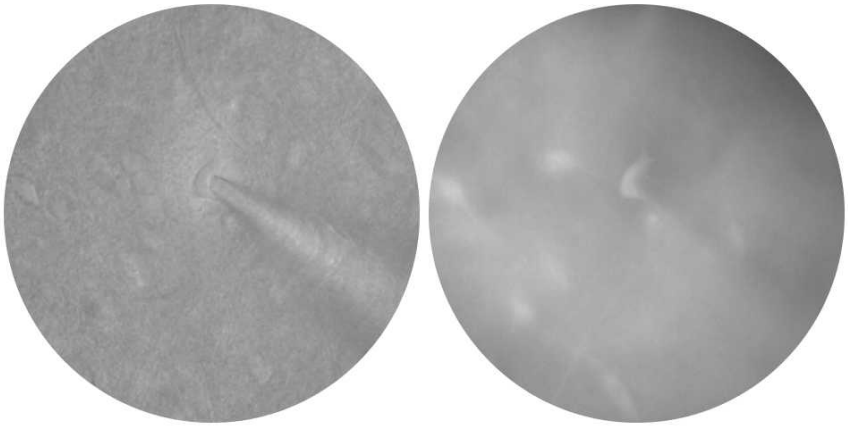
ACSF constantly equilibrated with 95% O2 and 5% CO2. Perfuse the solution at a flow rate of 2 mL/min by means of a peristaltic pump (PPS2, MultiChannel Systems) and warm to 32-34°C using a feedback-controlled in-line Peltier heater (Scientifica) for the length of the experiment.

- To visualise the target neurons within your area of interest, you can use a typical patch-clamp electrophysiology set up. You can locate the area of interest using a 4x objective (PLN, 0.1 numerical aperture, 18.5 mm working distance, Olympus) and visualise neurons with oblique illumination on an upright SliceScope Pro 1000 (Scientifica) using a 60x water-immersion objective (LUMPlanFLN, 1.0 numerical aperture, 2 mm working distance, Olympus) and a CMOS camera (Ximea MQ013MG-E2, Lambda Photometrics).
- Target neurons can be identified based on fluorescence from EYFP or tdTomato expression (depending on the transgenic line used) using LED illumination (pE-100, CoolLED) at wavelengths of 490 nm (EYFP animals) or 565 nm (tdTomato animals).



Images illustrating the identification of a target cell (left image) based on its fluorescence expression (right image).

8.2 Once the target cell has been located, proceed to the next steps:

- Take a freshly pulled pipette and backfill it with recording ACSF containing 2 U/µL recombinant RNase inhibitor. Unless you plan to obtain whole-cell recordings from the target cell before aspirating it, there is no need to use normal internal solution and blow potassium to all the cells while you approach the target cell.
- Insert the loaded pipette into the pipette holder of a patch-clamp headstage (EPC 800, HEKA), controlled by a motorised micromanipulator (PatchStar, Scientifica). Have a silver wire (0.35 mm diameter, GoodFellow, Cat. No. AG005145) coated with silver chloride (AgCl) present inside the pipette and in contact with the solution to act as a recording electrode, and either an Ag-AgCl pellet electrode (E206, 2.0 mm diameter, Warner Instruments, Cat. No. 641310) or a coiled AgCl coated silver wire immersed in the bath to act as ground electrode.
- Apply gentle positive pressure and, with the help of the micromanipulator, carefully bring the pipette into the bath and approach until you reach the surface of the slice. Position the tip of the pipette as close as possible to the point where the target cell is located.
- From the moment the pipette enters the bath, use a test pulse to measure the pipette resistance: you should aim for 1-2 MΩ.
- Prior to aspiration, switch from the 60x to the 4x objective and record the anatomical location of the neuron within you area of interest by capturing an image. The tip of the pipette should mark where your target cell is and can be used to obtain the coordinates within the common coordinate framework (see image registration section for more details).
- Once the image with the anatomical location has been acquired, switch back to the 60x objective and proceed to aspirate the cell.

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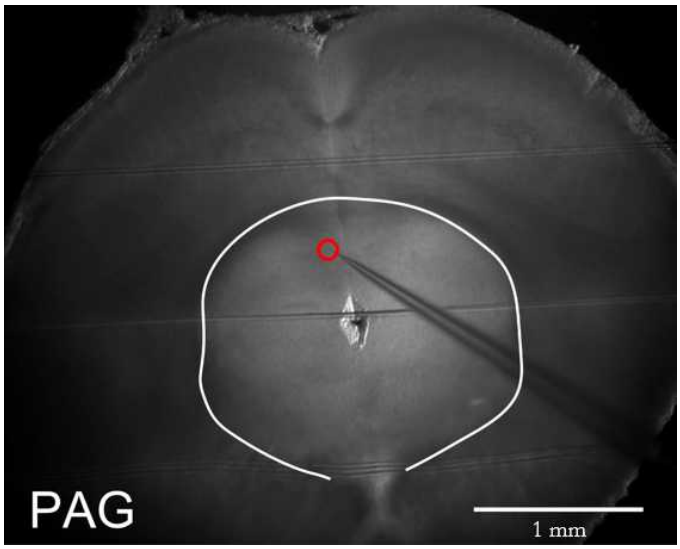

Example of an image obtained with a 4x objective prior to aspiration.
Red circle indicates the tip of the pipette, located just above the target cell.

8.3
- Approach the soma of the target cell with positive pressure applied to the pipette to prevent any dirt or unwanted tissue from being aspirated.
- Carefully position the tip in gentle contact with the cell membrane of the target cell and confirm it is the correct cell by checking its fluorescence expression one last time.
- Release the positive pressure and apply gentle suction to aspirate the cell into the tip of the glass pipette. If possible, acquire a video of the procedure for future reference.
- Once the cell has been aspirated, cease the suction and replace by minimal positive pressure while you carefully retract the pipette from the slice tissue. This should minimise aspiration of any surrounding tissue and processes from unwanted cells.
- Visual confirmation of successful single-cell isolation can be obtained by examining the pipette tip, where aspirated cells typically remained attached (see example video below).

Image sequence illustrating the isolation of a target cell by visually guided aspiration with a patch pipette.

8.4
- Retract the pipette containing the aspirated cell all the way out of the bath.

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- Detach the pipette from the holder and break its tip into the bottom of a 0.2 mL PCR tube containing 4 µL of lysis buffer, which was kept on ice until use.
- Once broken, quickly pull out the pipette from the tube to avoid drawing the lysis buffer and the sample back into it by capillary action.
- Close the tube and vortex for 10 seconds. Spin down (700g for 10 seconds at room temperature) to collect the liquid at the bottom of the tube and immediately place on dry ice to flash freeze the sample.
- Carry on with cell collection.

8.5 **[Example video of aspiration process]**

8.6

- Once cell collection is complete, samples can be stored at -80°C for several weeks until cDNA synthesis.

Smart-seq2 | cDNA synthesis 3h

9 [Batch and experimental design - in advance]

Whenever possible, consult a bioinformatician when planning and designing your experiments to ensure you avoid the common pitfalls and confounding factors. Having a good experimental design will ensure you can use the data to address the biological questions you have in mind, while circumventing the problems arising from batch effects.

The processing of different batches of samples is often subject to uncontrollable differences, e.g., changes in operator, differences in reagent quality, etc. This results in systematic differences in the observed gene expression in cells from different batches, which are often referred to as “batch effects”. Batch effects are problematic as they can be major drivers of heterogeneity in the data, masking the relevant biological differences and complicating interpretation of the results.

The approach followed in this protocol means that all the cells collected on a given experimental day will come from the same animal. Thus, if collecting (1) different cell-types or (2) one cell-type from different brain areas or different subdivisions from the same area in the same animal, it is critical to equally sample each variable in a pseudo-randomised order. If using transgenic animals and thus collecting different cell-types on different days (e.g. collecting cell-type A from animal 1, and cell-type B from animal 2), it is critical to account for that when processing the samples.

Ideally, if you are able to process 20 samples at once (what would be one processing batch), you should make sure that the sample distribution equally covers different cell-types, animals, and experimental days. Furthermore, each animal could be treated as a separate batch in their own right, reflecting (presumably uninteresting) biological differences due to genotype, age, sex, or other factors that are inherent to the experimental design. What you want to avoid is that one processing batch contains only 20 samples from the same cell-type and animal, as this would mean that these three variables (batch, cell-type, animal) are confounded and you won't be able to know which one is driving the biological effects on your data. Finally, it is a good idea to always add positive controls (e.g. ERCC spike-ins) and negative controls (empty samples or pipette tips that went into the slice but did not contain a cell) in each processing batch.

10 [Reverse Transcription ~3h]

For this and the following incubation steps, use a thermal cycler with a heated lid set to 105°C.

10.1 Clean the hood with RNaseZap and DNA-OFF solutions before setting up the working plates.

- Turn on the UV light for 20 mins, you can have the pipettes and other tools in there during that time.

Citation: Oriol Pavon Arocas, Sarah F. Olesen, Tiago Branco (08/11/2021). Visually guided aspiration of fluorescently labelled single neurons from acute midbrain slices followed by Smart-seq2. https://dx.doi.org/10.17504/protocols.io.bhfxj3pn

- Spray the pipettes and scissors (used to cut PCR tubes) with RNaseZap.
- Take an autoclaved beaker and put a plastic bag in it to collect used pipette tips.

Thaw the reagents needed for the reverse transcription (RT) mix. Calculate the volume you will need for the number of samples you want to process. For one sample, you will need 5.70 µL of RT mix (see below for exact volumes). Prepare enough solution for 1 extra sample, just to be safe and account for any volume lost during aliquoting. For each sample, you will need:

- 0.5 µL SuperScript II reverse transcriptase (200 U/µL).
- 0.25 µL recombinant RNase inhibitor (40 U/µL).
- 2 µL SuperScript II first-strand buffer (5x).
- 0.5 µL DTT (100 mM).
- 2 µL betaine (5 M).
- 0.06 µL MgCl2 (1 M).
- 0.1 µL template switching oligonucleotide (100 µM).
- 0.29 µL nuclease-free water.

10.2 Prepare the RT mix for all reactions plus one extra (to account for volume lost during pipetting).

- [!] Thaw all the reagents in advance and assemble the RT mix before performing the incubation step.
- Remember to use the small pipette tips that go with the appropriate volume, and always do the larger volumes first.

If you are processing 25 samples, prepare enough solution for 26 samples as follows:

- 13.00 µL SuperScript II reverse transcriptase (200 U/µL).
- 6.50 µL recombinant RNase inhibitor (40 U/µL).
- 52.00 µL SuperScript II first-strand buffer (5x).
- 13.00 µL DTT (100 mM).
- 52.00 µL betaine (5 M).
- 1.56 µL MgCl2 (1 M).
- 2.60 µL template switching oligonucleotide (100 µM).
- 7.54 µL nuclease-free water.

10.3
- Take the samples from the -80°C freezer and incubate at 72°C for 3 minutes in the thermal cycler.
- Immediately put the tubes back on ice (keeping samples on ice reduces secondary structure formation).
- Spin down the samples (700g for 10 seconds at room temperature) to collect the liquid at the bottom of the tubes, and immediately put them back on ice.
- The oligo-dT primers are now hybridized to the poly(A) tail of the mRNA molecules.

10.4 With the samples on ice, carefully add the RT mix. Each sample should roughly contain 4.3 µL: 4 µL from the lysis buffer and 0.3 µL from the aspirated cell and solution in the tip of the pipette.

- Bring up each sample containing approximately 4.3 µL lysis reaction to a volume of 10 µL by adding 5.7 µL reverse transcription master mix. **[!]** Do NOT pipette up and down.
- Spin down the samples (700g for 10 seconds at room temperature) to collect the liquid at the bottom of the tubes, and immediately put them back on ice.

10.5 To obtain cDNA from the single-cell lysates, the mRNA of each sample needs to be reverse transcribed by subjecting the samples to the following program in the thermal cycler:

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Cycle	Temperature (°C)	Time
1	42	90 min
2-11	50	2 min
	42	2 min
12	70	15 min
13	4 or 10	Hold

Reverse transcription program.

Smart-seq2 | cDNA preamplification 3h

11 [PCR preamplification]

For each sample, you will need 25 µL of PCR mix:

- 10 µL product of the reverse transcription reaction (previous section).
- 12.5 µL KAPA HiFi Hotstart ReadyMix (2x, KAPA Biosystems).
- 0.25 µL ISPCR primer (10 µM).
- 2.25 µL nuclease-free water.

11.1 Prepare enough PCR mix for all the samples plus one extra (to account for volume lost during pipetting). If processing 25 samples, prepare enough solution for 26 samples by mixing:

- 325.00 µL KAPA HiFi Hotstart ReadyMix (2x, KAPA Biosystems).
- 6.50 µL ISPCR primer (10 µM).
- 58.50 µL nuclease-free water.

11.2 For each sample:

- Add 15 µL of PCR mix to the tube containing the 10 µL from the reverse transcription reaction.
- Vortex the tubes for 10 seconds to mix, and then spin them down (700g for 10 seconds at room temperature) to collect the liquid at the bottom of the tubes.

11.3 To amplify the cDNA, subject the samples to the following PCR preamplification program in the thermal cycler with a heated lid set to 105°C:

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Cycle	Temperature (°C)	Time
1	98	3 min
2-19	98	20s
	67	15s
	72	6 min
20	72	5 min
21	4	Hold

PCR preamplification program.

11.4

Samples can be stored at -20°C or -80°C until purification (for 6 months or longer).

Smart-seq2 | cDNA purification and quality control 6h

12 [Purification ~3h]

This step can be done without the hood. Spray and clean the lab bench with DNA-OFF.

12.1
- Equilibrate the Ampure XP beads at room temperature for 15 minutes, and then vortex well for several seconds. Ensure the solution is well mixed and appears homogeneous.
- While the beads equilibrate, prepare a fresh solution of 80% ethanol. Ethanol absorbs moisture from the environment and the concentration can change over time, so it is critical that a fresh solution is made each time.
- You will need a 1:1 ratio of beads:sample. Each sample contains 25 µL of the preamplification reaction, so you will need 25 µL of beads per sample. Aliquot out the total volume of beads you will need plus enough for two extra samples.

12.2
- Add 25 µL Ampure XP beads (1:1 ratio) to each sample and mix by carefully pipetting up and down until the solution is homogeneous. **[!] Do not spin the solution** (the beads will go to the bottom of the tube if you do that).
- Incubate the samples for 8 minutes at room temperature to let the cDNA bind to the beads.
- Next, place the samples on a magnetic stand until the solution is clear and the beads are collected at the bottom of the tube.

12.3
- With the samples still on the magnetic stand, carefully remove the liquid without disturbing the beads (usually around ~45 µL can be removed).
- Wash the beads by adding 200 µL 80% ethanol and incubating for 30 seconds. Remove the ethanol without disturbing the beads.
- Repeat the washing step once more.
- To remove any trace of ethanol, dry the beads completely by leaving at room temperature for 5 minutes or until a small crack appears on the surface of the beads. Avoid overdrying the beads, as this will make it harder to resuspend them. Cover the samples during this step to protect them from any source of contamination or air flow that may lead to cross-contamination between samples close by.

- Add 17.5 µL elution buffer solution to each sample. Mix ten times by pipetting up and down to

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12.4 resuspend the beads.

- Incubate **off** the magnetic stand for 2 minutes.
- Place the samples back on the magnetic stand and leave for 2 minutes or until the solution is clear and beads have accumulated at the bottom of the tube.
- To ensure minimal bead carryover, collect 15 µL of the supernatant containing the purified cDNA from your samples without disturbing the beads. Transfer to a fresh PCR tube. Avoid aspirating the whole volume of elution buffer solution - leaving 2.5 µL in the well ensures that bead carryover is kept to a minimum. Importantly, bead carryover will reduce the quality of your cDNA library, and it is observable in the Bioanalyzer traces.

12.5

Purified samples can be stored at -20°C or -80°C until quality checks are performed.

13 [Quality control ~3h]

Three independent measures can typically be used to quality check your samples.

13.1 [Sample purity with NanoDrop spectrophotometer]

Sample purity can be estimated using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The ratio of absorbance at 260 and 280 nm ($A_{260/280}$ ratio) is routinely used to assess the purity of RNA and DNA preparations.

- A DNA sample is considered pure if it has a $A_{260/280}$ ratio of ~1.8, whereas an $A_{260/280}$ ratio of less than 1.8 is indicative of the presence of unwanted compounds such as proteins.
- Samples with an $A_{260/280}$ ratio below 1.8 can be discarded if the other quality checks also fail or can be put through a new round of purification and checked again.

13.2 [cDNA concentration with Qubit fluorometer]

cDNA concentration of the purified samples can be determined using the Qubit dsDNA High-Sensitivity Assay Kit (Invitrogen) in a Qubit 2.0 fluorometer (Life Technologies). The fluorometer measures the concentration of RNA or cDNA bound to a fluorescent dye.

- Samples with a Qubit-measured concentration below 1 ng/µL can be discarded if the other quality checks also fail.

13.3 [cDNA size distribution with Bioanalyzer]

The size distribution of the cDNA of each sample can be determined using Agilent High-Sensitivity DNA chips (Agilent Technologies) run in an Agilent 2100 Bioanalyzer (Agilent Technologies).

- Bioanalyzer profiles with a peak in the 1500-3000 bp range, a small number of fragments below 500 bp, and small number of primer dimers indicate that the majority of cDNA has been produced from intact mRNA and the resulting cDNA is of good quality.

13.4

Samples that pass the NanoDrop and Qubit quality checks and show good Bioanalyzer profiles can be deemed of good quality and sequenced.

Purified and QCed samples can be stored at -20°C until sequencing (for 6 months or longer).

cDNA library construction and sequencing 4w

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14 **[Tagmentation, library construction, and DNA sequencing]**

These steps were done externally by the Barts London Genome Centre at the Blizard Institute (Barts and The London School of Medicine and Dentistry, Queen Mary University of London). The time until receiving the data will vary depending on the number of projects on the cue, and it could take more or less than 4-6 weeks depending on the facility you use or on whether your institute has a core facility that can do it for you quicker.

14.1 Purified samples were stored at -20°C until enough were obtained for sequencing. Samples were transported on dry ice to the sequencing facility and stored at -20°C until use. Prior to library generation, cDNA samples were quantified using the Qubit dsDNA High-Sensitivity Assay Kit and the Qubit 2.0 Fluorometer (Life Technologies); and a subset of cDNAs were checked for quality using the Agilent 2200 Tapestation (Agilent Technologies).

14.2 Libraries were prepared using the Illumina Nextera XT Sample Preparation Kit (Illumina Inc.) with an input of 150 pg of cDNA per sample. Resulting libraries were checked for average fragment size using the Agilent D1000 ScreenTape (Agilent Technologies) and were quantified using the Qubit dsDNA High-Sensitivity Assay Kit (Invitrogen). Equimolar quantities of each sample library were pooled together and 75 bp paired-end reads were generated for each library using the Illumina NextSeq 500 High-output sequencing kit, with a target sequencing depth of ~4.16 M reads per sample.

15 **Important considerations when determining the sequencing depth for your experiment:**

[From Wang et al. - Nature Reviews Genetics 2009 - RNA-seq: a revolutionary tool for transcriptomics]

- The number of expressed genes detected by RNA-Seq reaches 80% coverage at 4 million uniquely mapped reads, after which doubling the depth merely increases the coverage by 10%.

[From Streets & Huang - Nature Biotechnology 2014 - How deep is enough in scRNAseq?]

- Single-cell RNA-seq studies usually involve a trade-off between the number of samples analyzed and the number of RNA transcripts sequenced per cell, or sequencing depth, that can be achieved.
- Pollen et al. demonstrate accurate and reliable classification of cell types at a sequencing depth of only 50,000 reads per cell.
- This sequencing depth can be useful to identify cell-types objectively and discover novel biomarkers.

[From Pollen et al. - Nature Biotechnology 2014 - Low-coverage scRNAseq reveals cellular heterogeneity]

- By capturing 301 single cells from 11 populations using microfluidics and analyzing single-cell transcriptomes across downsampled sequencing depths, they demonstrate that shallow single-cell mRNA sequencing (~50,000 reads per cell) is sufficient for unbiased cell-type classification and biomarker identification.
- They sequenced libraries from single cells at high coverage (~8.9 M reads per cell) and used the results as a reference to explore the consequences of reduced sequencing depth.
- Transcripts expressed at low levels (between 1 and 10 TPM-transcripts per million) were not detected when using shallow sequencing.

[From Lei et al. - Gene 2015 - Diminishing returns in NGS transcriptome data]

- They selected 36 RNA-seq datasets, each with more than 20 million reads from six widely-used model organisms: Saccharomyces cerevisiae, Homo sapiens, Drosophila melanogaster, Caenorhabditis elegans, Mus musculus, and Arabidopsis thaliana, and investigated statistical correlations between the sequencing depth and the outcome accuracy.
- Their results indicated that as low as one million reads can provide the same sequencing accuracy in transcript abundance (r = 0.99) as 30 million reads for highly-expressed genes in all six species.

[From Svensson et al. - Nature Methods 2017 - Power analysis of scRNA-seq experiments]

- They compared 15 protocols computationally and 4 protocols experimentally for batch-matched cell populations, in addition to investigating the effects of spike-in molecular degradation. Our analysis provides an integrated framework for comparing scRNA-seq protocols.
- They defined the **sensitivity** of a method as the minimum number of input RNA molecules required for a spike-in control to be confidently detected (also known as the lower molecular-detection limit, for a given sequencing depth), and defined the **accuracy** as how close the estimated relative abundance levels were to the known abundance levels of input molecules. High sensitivity permits the detection of very weakly expressed genes, whereas high

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accuracy suggests that detected variations in expression reflect true biological differences in mRNA abundance across cells, rather than technical factors.

- **Accuracy** does not strongly depend on sequencing depth (accuracy becomes saturated at as few as 250,000 reads). However, **sensitivity** is critically dependent on sequencing depth, and it saturates at 4.5 million reads per sample (however, there is not much improvement from 1 million to 4.5 million reads, whereas the difference is significant from 100,000 to 1 million reads).
- They recommend 1 million reads per sample as a good target for saturated gene detection.
- Both sensitivity and accuracy are closely dependent on the scRNA-seq protocol used to generate the data. Protocols with high sensitivity are more suitable for analyzing weakly expressed genes, or for gaining additional insights into subtle gene expression differences affecting individual cell states, but may be less suitable for other scenarios

[Ziegenhain et al - Molecular Cell 2017 - Comparative analysis of scRNA-seq methods]

- They evaluate six prominent scRNA-seqmethods: CEL-seq2, Drop-seq, MARS-seq, SCRB-seq, Smart-seq, and Smart-seq2.
- One type of technical variable is the **sensitivity** of a scRNA-seq method (i.e., the probability to capture and convert a particular mRNA transcript present in a single cell into a cDNA molecule present in the library). Another variable of interest is the **accuracy** (i.e., how well the read quantification corresponds to the actual concentration of mRNAs), and a third type is the **precision** with which this amplification occurs (i.e., the technical variation of the quantification).

[!] Set the sequencing depth a bit higher of what you aim for:

Importantly, once you have decided on the sequencing depth and number of cells that is appropriate for your experiment, try to account for the loss of useful reads that usually occurs during any sequencing run.

[From Picelli et al. Nature Protocols 2014 - Full-length RNA-seq from single cells using Smart-seq2]

- When sequencing 50-bp single-end reads, they find that normally 60% of reads map uniquely to the genome (20% multimapping and 20% with no match); of the uniquely mapping reads, >60% of the reads map to annotated RefSeq exons, 20% intronic and 20% intergenic, but these values depend on the completeness of the gene annotations.

This values suggest that if you go for a sequencing depth of 1 million reads per sample, around 600,000 will uniquely map to the genome, from which 360,000 will ultimately map to annotated exons (and will therefore be useful to your experiment). Furthermore, sequencing facilities tend to be a bit conservative on the total amount of cDNA they input on a given sequencing run to avoid saturating the system, which usually means that you will never use up 100% of the available reads in the kit you choose. Overall, this means that only a fraction of the reads you think you will get are used, and only a fraction of the reads used will give you useful data.

We observed a similar trend in our experiments. We used the Illumina NextSeq 500 High-output sequencing kit, which provides up to 400 million reads that are spread between the 96 samples we sequence together. This approach should theoretically result in a sequencing depth of ~4.16 million reads per sample. However, the average number of reads uniquely mapping to annotated exons was ~2.2 million reads per sample (~53% of the target), out of which ~13% corresponded to Mitochondrial genes, Ribosomal genes, ERCC spike-ins, or TSO concatamers. The rest was probably lost in multimapping reads, reads with no match on the genome, and reads not used due to inputting an amount of cDNA below the actual limit of the kit and sequencing system. Overall, we achieved ~1.9 million uniquely mapping reads per sample, a value that according to most studies is beyond the saturation point of gene detection for accuracy and sensitivity.

Image registration

16 The recent development of tools makes it possible to obtain the approximate coordinates of each isolated cell. This can be leveraged for visualisation purposes and data analysis. To achieve this, you need to calibrate the pixel size of the images you obtained to record the anatomical location of each cell before aspiration. You can use a 1 mm stage microruler with 10 µm divisions to do this. To facilitate registration, each image should contain the main landmarks of your area of interest and the pipette position right above the cell you are about to aspirate. It is best to capture this image once the target cell has been identified and the pipette has been loaded and position at the surface of the slice, but before penetrating the tissue and aspirating the cell (once the cell has been aspirated, it should be transferred to the PCR tube with lysis buffer as soon as possible).

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16.1 [Image registration with Sharp-Track]

Images with the anatomical location of each isolated neuron can be registered to the Allen Mouse Brain Common Coordinate Framework using the Slice Histology Alignment, Registration, and Probe Track analysis tool (SHARP-Track) as described in the GitHub wiki (https://github.com/cortex-lab/allenCCF/wiki). You will need to calibrate the pixel size of your images beforehand. For more information on the Mouse Brain Common Coordinate Framework and SHARP-Track, see:

- Q. Wang, S. L. Ding, Y. Li, et al. Cell 2020. The Allen Mouse Brain Common Coordinate Framework: a 3D Reference Atlas. DOI: https://doi.org/10.1016/j.cell.2020.04.007
- P. Shamash, M. Carandini, K. Harris, N. Steinmetz. bioRxiv 2018. A tool for analyzing electrode tracks from slice histology. DOI: https://doi.org/10.1101/447995
- SHARP-Track GitHub repository: https://github.com/cortex-lab/allenCCF

16.2 [3D renderings of single-cell coordinates with Brainrender]

Once you have obtained the coordinates within the Common Coordinate Framework for each aspirated cell you can use *brainrender* to generate 3D renderings to visualise all the cells and selected metadata. See following references for more information on Brainrender:

- F. Claudi, A. L. Tyson, L. Petrucco, T. W. Margrie, R. Portugues, T. Branco. eLife 2021. Visualizing anatomically registered data with Brainrender. DOI: https://doi.org/10.7554/eLife.65751
- F. Claudi, A. L. Tyson, L. Petrucco, T. W. Margrie, R. Portugues, T. Branco. bioRxiv 2020. *Brainrender*: a python-based software for visualizing anatomically registered data. DOI: https://doi.org/10.1101/2020.02.23.961748
- Brainrender GitHub Repository: https://github.com/brainglobe/brainrender

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