Chapter X

Combining whole-cell patch-clamp techniques with single-cell RNA sequencing

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Abstract

To understand how the brain functions we need to understand the properties of its constituent cells. Whole-cell patch-clamp recordings of neurons have enabled studies of their intrinsic electrical properties as well as their synaptic connectivity within neural circuits. Recent technological advances have now made it possible to combine this with a sampling of their transcriptional profile. Here we provide a detailed description how to combine whole-cell patch-clamp recordings of neurons in brain slices followed by extraction of their cytoplasm suitable for single-cell RNA-sequencing and analysis.

Key words: Single-cell RNA sequencing, Electrophysiology, Patch-clamp, Brain slice, Gene expression

1 Introduction

Whole-cell patch-clamp recordings both *in vitro* and *in vivo* have provided great insight into the electrical diversity that exists amongst neurons in the brain. Similarly, recent high-throughput single-cell RNA-sequencing efforts have provided great insights into the transcriptional diversity that exists amongst neurons of the brain [4, 6, 8, 9, 10, 11, 13]. Such efforts have provided a transcriptomic-based taxonomy of cell types e.g. in cortical structures comprising over 100 distinct cell classes [10, 11, 13]. These recent endeavours have often used high throughput approaches using suspensions of cells [4, 5, 9, 10, 11] or nuclei [8] derived from dissociated brains, which does not allow for the characterization of electrical properties of neurons. Previous approaches combining whole-cell patch-clamp recordings with genetic analysis often used RT-PCR and was limited to looking at predetermined subsets of genes [12]. To provide an unbiased assessment of gene expression as well as being able to interrogate the electrical and circuit properties of mature neurons we and other groups have used an approach that combines whole-cell patch-clamp electrophysiology with subsequent single-cell RNA sequencing [1, 3, 6]. In particular, we used *in utero* electroporation techniques to fluorescently label cortical neurons derived from distinct progenitor pools and used this approach to characterize their intrinsic and circuit properties as well gene expression [3]. Here we provide a detailed step-by-step instruction on how to progress from making acute brain slices to performing whole-cell patch-clamp recordings under conditions that allow for the successful extraction and processing of the mRNA from the cytoplasm of the patched neuron (Fig. 1).

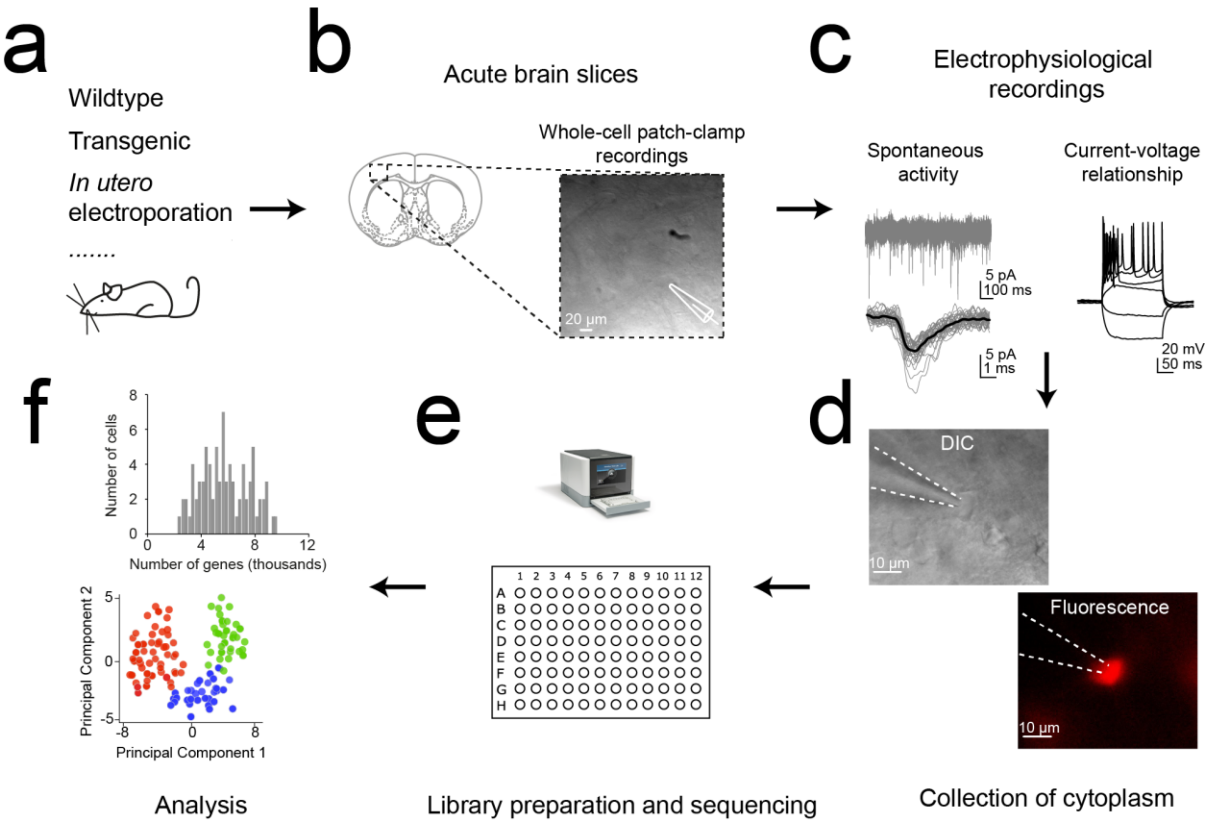

Fig. 1 From whole-cell patch-clamp recordings to single-cell RNA sequencing workflow (a, b) Acute brain slices were prepared as described in the step **3.2** and transferred to a storage chamber. Slices can come from any wildtype or transgenic animal and in our case came from animals that had undergone *in utero* electroporation to label neurons derived from distinct progenitor pools (c) Whole-cell patch-clamp recordings were performed from neurons in somatosensory cortex and included recordings of spontaneous activity as well as current-voltage relationships. (d) After recordings the cytoplasmic content of the neuron was extracted as described in the step **3.4**. The example shown here is a tdTomato expressing Layer 2/3 pyramidal neuron. Top image is a Dodt contrast image for visualisation while patching and the bottom image (red) is in fluorescent light. (e) The extracted cytoplasmic content was transferred to a 96 well plate. Further slots were filled in the same manner as described above and once the plate was completed, cDNA synthesis, library preparation and sequencing was performed as described in step **3.5**. (f) The sequencing and analysis can provide information on the differential expression of thousands of genes.

2 Materials

2.1 Solutions

1. Prepare all solutions under RNase-free conditions (*see* **Note 1**) in deionised water (dH_2O) treated with 0.1% Diethyl pyrocarbonate (DEPC) and autoclaved or separately purchased DNase/RNase-Free distilled water can be used. Store all the solutions at 4 ^oC, unless mentioned otherwise.
2. Extracellular cutting solution: 230 mM D-sucrose, 26 mM $NaHCO_3$, 3.5 mM KCl, 10 mM glucose, 1.25 mM NaH_2PO_4, 1.3 mM $CaCl_2$, 2.6 mM $MgCl_2$, pH 7.2 – 7.3 and 310 mOsmol/l, bubbled with carbogen (95% oxygen, 5% carbon dioxide) and chilled on ice for at least 45 min. prior to use (*see* **Note 2**).
3. Extracellular recording solution: 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM D-glucose, pH 7.2-7.3; osmolarity, 300-310 mOsmol/l, continuously bubbled with carbogen and for at least 30 mins before the start of electrophysiological recordings.
4. Intracellular solution (current clamp): 123 mM potassium gluconate, 12 mM KCl, 10 mM HEPES, 0.2 mM EGTA, 4 mM MgATP, 0.3 NaGTP, 10 mM sodium phosphocreatine, 1U/µl recombinant RNAse inhibitor, pH 7.2-7.3; osmolarity, 290-295 mOsmol/l (*see* **Note 3**). Add the RNAse inhibitor on the day of recording. The EGTA in the intracellular solution will scavenge free calcium and reduce potential RNAse activity [2].
5. Smart-seq2 cell lysis buffer: 1.3 µl of 0.2% v/v Triton X-100 containing 2U/µl RNase inhibitor, 1µl of 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH) and 1µl of 100 µM Oligo-dT_{30}VN primer (*see* **Note 13**).

6. Reverse transcription mix: 10U/µl SuperScript IV reverse transcriptase, 5mM DL-Dithiothreitol (DTT), 1X SuperScript IV Buffer, 1M Betaine, 1 µM locked nucleic acid-Template-switching oligonucleotide (LAN-TSO) (*see* **Note 14**), 1U/µl recombinant RNAse inhibitor, 6mM $MgCl_2$.
7. cDNA amplification PCR mix: 1X KAPA HiFi hotstart ready-mix, 0.1 µM IS PCR oligos (5'-AAGCAGTGGTATCAACGCAGAGT-3').

3 Methods

3.1 Preparation for RNAse-free workspace

1. All the chemicals and glassware should be RNAse-free and kept separately as "RNA only". Any handling of equipment should be done using gloves to minimize contamination.
2. Empty the electrophysiology patch-clamp rig of all non-essential pieces of equipment (Fig. 2) and replace all existing tubing on the perfusion system. During the period of experiments involving cytoplasm extraction, clean the rig regularly and thoroughly by spraying with RNaseZAP and wiping the surfaces using RNase Decontamination Wipes followed by final spraying with DEPC-treated dH_2O and a wipe using sterile RNAse-free paper towels.
3. Prepare a dedicated space in the lab that should be used to process all RNA related work and clean this space once a week by following the same cleaning protocol described in the previous step.
4. Before making brain slices, clean the vibratome using a similar procedure. Briefly, spray with RNAaseZAP and surface clean using RNase Decontamination Wipes and finally spray with DEPC-treated

dH_2O and wipe clean with RNAse-free paper towel. Make sure to clean the metal/ceramic blades and brain stage also.

5. The storage chamber to store brain slices should be cleaned the day before use by spraying with RNAaseZAP and adding a 10% bleach solution. Leave the solution mix for 5 minutes to prevent any bacterial/fungal growth. Subsequently rinse the chamber out with DEPC-treated dH_2O and leave to air dry, ready for use the next day.

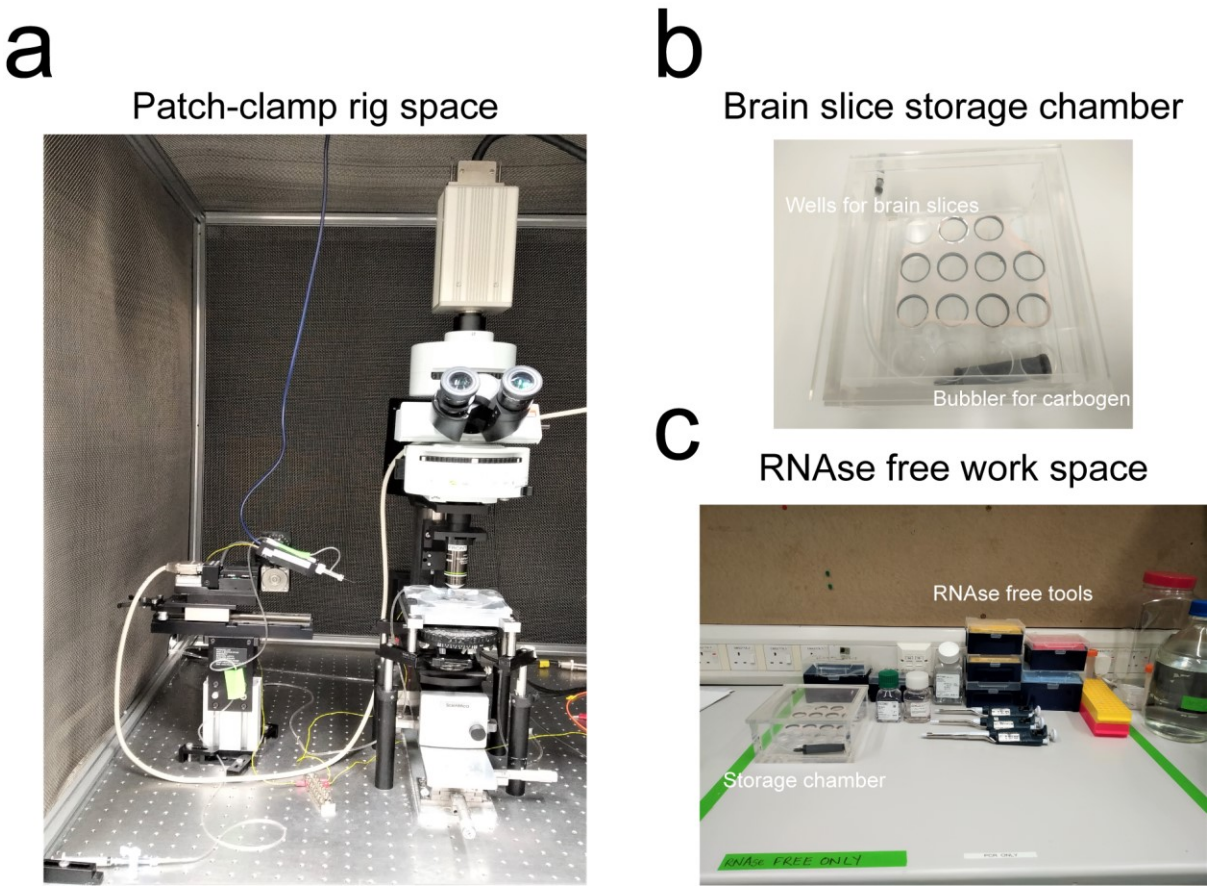

Fig. 2 Electrophysiology rig space and equipment (a) The rig space should be clean and kept clear of any unnecessary items. (b) Acute brain slice storage chamber containing bubbler for carbogen. (c) RNAse free and tidy dedicated workspace for sample processing.

3.2 Preparation of acute brain slices

1. Deeply anaesthetise the rat / mouse using 3-4% isoflurane and decapitate rapidly. Drop the head directly in ice-cold cutting solution (*see* **Note 2**). Wait for approximately 30 seconds while the head is still submerged in the cutting solution to help it cool down.
2. Dissect out the brain and keep this in the ice-cold cutting solution.
3. Glue the brain on the vibratome stage (*see* **Note 4**).
4. Make 300 - 400 µm brain slices using the vibratome while the brain is continuously submerged in ice-cold cutting solution (*see* **Note 5**).

5. The vibratome setting for acute brain slicing in our hands is 0.5-0.6 mm/s forward speed, 0.9 mm amplitude and 90 Hz frequency.
6. Immediately transfer the slices to the storage chamber with recording solution maintained at 35^{o}C for 30 minutes (*see* **Note 6**). Move the chamber to room temperature afterwards. Let the slices rest for at least 20 minutes before starting recordings and use the brain slices within the next 4 hours when collecting cytoplasm to avoid potential transcriptional changes that might occur with prolonged storage.

3.3 Electrophysiological recordings

1. Transfer one slice to the clean patch-clamp recording setup with oxygenated solution flowing at 3 ml / min.
2. Neurons can be visualised at 20x and 40x using video assisted infrared differential interference contrast (DIC) or Dodt contrast imaging (Fig. 3).
3. Identify the target neuron as standard for whole-cell patch-clamp recordings; taking into consideration the size, shape and depth of the neuron (**see Chapter X**).
4. It is important to backfill the patch pipette with approximately 1 µl intracellular solution just prior to patching from a neuron (Fig. 4) to avoid evaporation of the intracellular solution. The resistance of the patch pipette should be between 3-4 MΩ.
5. Make sure that the length of the silver chloride wire in the patch electrode is precise and just long enough to encounter the intracellular solution (Fig. 4).
6. Approach the cell with positive pressure (~10 mbar) (*see* **Note 7**). After contacting the cell, achieve a giga-seal configuration by releasing the

positive pressure combined with gentle suction. Voltage-clamp the cell at -70 mV.

7. Wait for a minute before breaking open the cell by applying sudden negative pressure (*see* **Note 8**).
8. Perform whole-cell patch-clamp recordings to assess electrical and circuit properties and any other parameters of interest.

3.4 Cytoplasm collection for RNA-sequencing

1. After finishing the electrophysiological recordings (*see* **Note 9**) aspirate the entire cellular contents of the cell into the patch pipette by applying light suction sustained over the period of 1-2 minutes (*see* **Note 10**)
2. The transfer of the cytoplasm should be confirmed by visualisation under DIC and/or fluorescent light. The patched cell should shrink completely and the cytoplasm along with nucleus should move inside the patch pipette (Fig. 3).
3. Equilibrate the pressure after completing the transfer of cellular content by releasing the negative suction and withdraw the patch pipette from perfusion chamber. It is crucial to equilibrate the pressure and make sure to lock the stopcock valve while taking the pipet out from the slice to avoid losing the extracted cytoplasm.
4. Expel the content of the patch pipette into an RNA-free microcentrifuge tube prefilled with ~3.3 µl Smart-seq2 cell lysis buffer.
5. This is a critical step and extra care should be taken. Apply strong positive pressure once the tip is located near the bottom of the microcentrifuge tube. To ensure that the content is transferred, break the

tip of the patch pipette near the side and bottom of the microcentrifuge tube (*see* **Note 11**).

6. Transfer the sample to dry ice immediately and store at -80^{o}C for further processing (*see* **Note 12**).

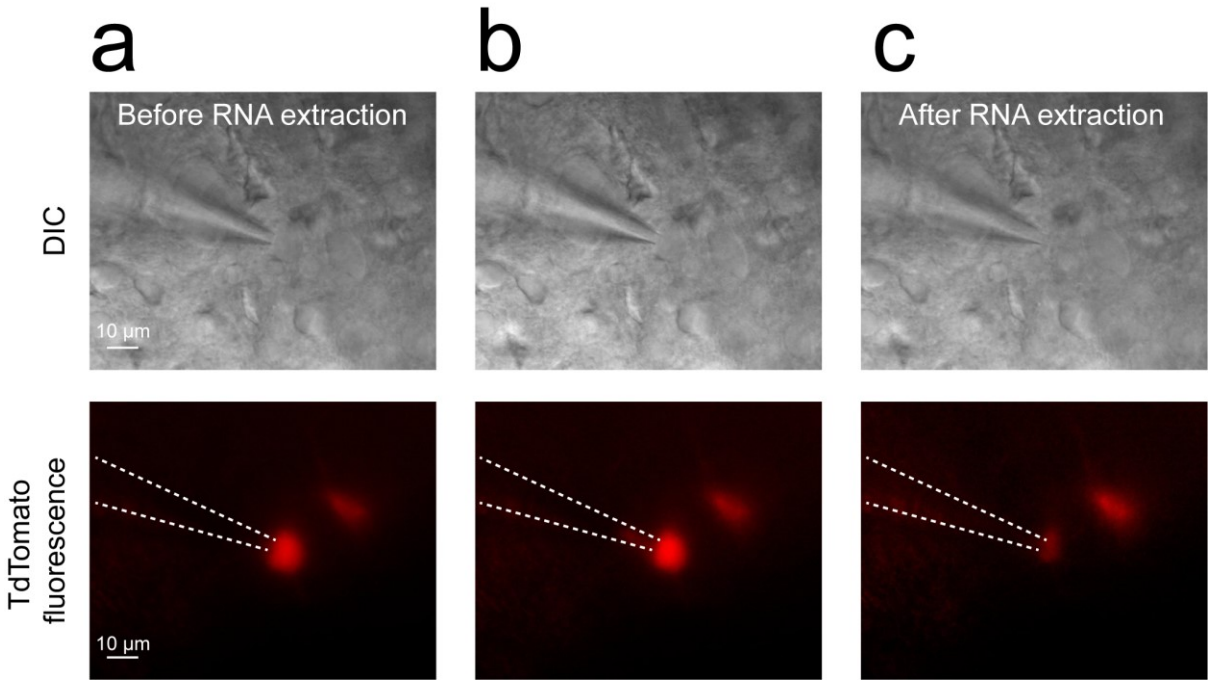

Fig. 3 Collecting cytoplasm from patched neurons **(**a**)** DIC and fluorescent (tdTomato) image while performing whole-cell patch clamp recording. **(**b**)** Extraction of cellular content (see step **3.4**). The fluorescent protein can be seen moving into the patch pipette along with cytoplasm. **(**c**)** DIC and fluorescent image after the extraction of cellular content.

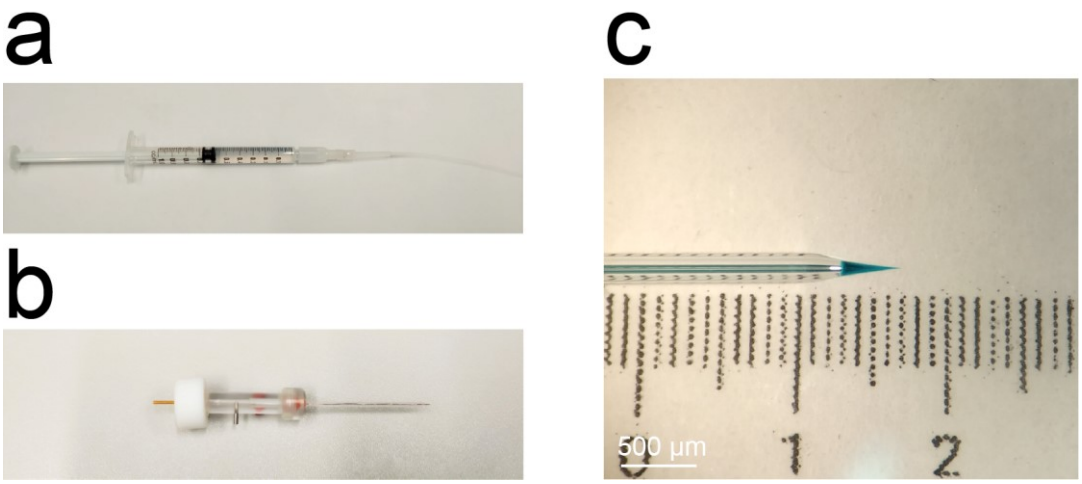

Fig. 4 Preparing glass recording pipets (a**)** To backfill the micropipette with internal solution a microloader tip was attached to a filled 1 ml syringe and intracellular solution was filtered through a 0.22 μm syringe filter. **(**b**)** Micropipette holder with relatively long silver wire. **(**c**)** Micropipette with ~1μl intracellular solution (fast green dye was added for demonstration purpose only).

7. Clean the silver wire before patching the next cell to get rid of previous mRNA. This is done through the usual steps as outlined above i.e. spray with RNAaseZAP, clean using RNase Decontamination Wipes and spray with DEPC-treated dH_2O and wipe clean with RNAse-free paper towel.
8. Now it is possible to record from the next neuron.
9. It is important to interleave the collection of neurons if one is comparing two different populations, for example to control for changes in expression related to storage of slices in the holding chamber or other factors such as time of day.

3.5 Single-cell RNA-sequencing

3.5.1 Reverse Transcription

1. Use Smart-seq2 protocol to convert the collected RNA sample into complementary DNA (cDNA)[7]. The steps are briefly described below.
2. Remove the samples from -80°C storage and centrifuged at 700*g* in a microcentrifuge for 30 seconds at 4°C and transfer the tubes on ice.

3. Set up the thermal cycler at 72 °C and incubate the collected RNA samples at 72 °C for 3 min. Transfer the tubes back on ice after completing the incubation. This step denatures the mRNA and help oligo-dT primer to hybridize with the poly(A) tail.

4. Prepare the reverse transcription (RT) master mix by combining the components listed in the table below. To avoid any shortage, prepare one extra reaction mix.

Components	Volume (µl)	Final concentration in 10 µl reaction mix
SuperScript IV reverse transcriptase (200 U/µl)	0.50	10 U/ µl
DTT (0.1 M)	0.50	5 mM
SuperScript IV Buffer (5X)	2.00	1X
Betaine (5 M)	2.00	1 M
LAN-TSO (100 µM)	0.1	1 µM
Recombinant RNAse inhibitor (40 U/µl)	0.25	1 U/ µl
$MgCl_2$ (1 M)	0.06	6 mM
Ultrapure nuclease free water	0.29	-
Total Volume	5.70	-

5. Add 5.70 µl of RT mix to each of the 0.2 ml microcentrifuge tube and mix gently three times to avoid any bubble formation. This will make up the total volume of 10 µl. Centrifuge the sample at 700*g* in a microcentrifuge for 10 seconds at 4°C and transfer the tubes on ice.

6. Transfer the reaction mix to a thermal cycler with a preheated lid and incubate the sample using the programme described in the table below.

Cycle	Temperature (°C)	Time
1	42	90 min
2–11	50	2 min

	42	2 min
12	70	15 min
13	4	Hold

7. At this stage, cDNA can be stored at 4 ^{o}C for further processing.

3.5.2 cDNA amplification

1. Mix the components listed in the table below to prepare a PCR master mix. It is always a good practice to prepare for a few extra reactions.

Components	Volume (µl)	Final concentration in 25 µl reaction mix
KAPA HiFi HotStart ReadyMix (2×)	12.50	1X
IS PCR primers (10 µM)	0.25	0.1 µM
Ultrapure nuclease free water	2.25	-
Total Volume	15	-

2. Add the 15 µl PCR master mix to the 10 µl cDNA sample obtained from the reverse transcription step. This will make for a final PCR reaction volume of 25 µl. Mix the sample and centrifuge at 700*g* for 10 s.

3. Set up the thermal cycler with the following programme and run the sample.

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

4. At this point we would send of the samples to a sequencing facility, which aided with further processing, quality check and sequencing following the Smart-seq 2 protocol [7]. Randomize the location of samples on the plate to avoid location effects.

4 Notes

1 RNAse-free conditions involve the use of glassware and tools that are completely RNAse free. Handling items with gloves and autoclaving prior to use and/or thorough cleaning by spraying with RNAaseZAP followed by a wipe down with RNase Decontamination Wipes and finally a spray with DEPC-treated dH_2O and a wipe with sterile paper towels guaranteed this.

2 Putting cutting solution in a -20^{o}C freezer for 10 minutes before cutting allows for a colder mixture containing ice. This seems to produce better and more viable slices especially when working with tissue from older animals (> 1 month).

3 Filter the solution using 0.22 μm syringe filter. Measure the osmolality before storing the intracellular solution in 500 μl tubes at -80 ^{o}C. Thaw one tube on the day of recording and use it the same day and discard anything left over. Check the osmolality again after adding the RNAse inhibitor and adjust by adding RNAse-free dH_2O. Do not refreeze the solution for future use.

4 For coronal brain sections containing cortical and subcortical regions the cerebellum is removed and the cut surface is glued down.

5 Based on experience of using both metal blades and ceramic blades we find that slices are better using reusable ceramic blades. However make sure to clean ceramic blades before every use to get rid of any potential RNAse activity.

6 Storing the brain slices at 35^{o}C for 30 minutes produced slices that had a clearer surface and improved visibility making it easier to find healthy neurons to patch.

7 A manometer is initially useful for this. Later on a fixed volume in a 1 ml syringe can also be used.

8 Approach the cell diagonally and not from the top as this would avoid any compression of the cell or tissue. When the patch pipette comes in proximity of the cell, a dimple should appear on the cell membrane because of the positive pressure. Apply negative pressure to achieve gigaseal configuration only after the appearance of dimple. Pull the patch pipette slightly backwards to relax the membrane before breaking in to achieve whole-cell configuration. Do not press on to the cell with patch pipette. Discard the cell if the health deteriorates or the membrane ruptures immediately after breaking in.

9 In our experiments it has been possible to record for up to 30 minutes in whole-cell configuration and still be able to successfully extract the cytoplasm.

10 It does not matter whether this is in current-clamp mode or voltage-clamp mode. Maintain visual inspection of the neuron to control how much suction needs to be applied. When the nucleus of the neuron gets stuck at the tip a longer period of suction might be needed or we often resorted to applying suction using a 2.5 ml syringe to increase the amount of suction.

11 Strong positive pressure should be applied before breaking the tip and not after. Best results are achieved when the patch pipettes are broken while continuously applying positive pressure.

12 Samples stored at -80 ^{o}C for 2 months were still usable and yielded good data.

13 The Oligo-dT$_{30}$VN (5'-AAGCAGTGGTATCAACGCAGAGTACT$_{30}$VN -3') primer specifically binds to all the RNA containing poly(A) tail. The

3’ end contains 30 thymine bases; A, C, or G is represented by ‘V’ and ‘N’ is any of the 4 nucleotides. Prepare a 100µM stock solution by dissolving the oligonucleotides in TE buffer. They can be stored in small aliquots (40-50 µl) at -20 ^{o}C for a year.

14 There are two riboguanosines (rG) and one locked nucleic acid (LAN)-modified guanosine (+G) at the 3’ end of template switching oligonucleotide (5’-AAGCAGTGGTATCAACGCAGAGTACATrGrG +G-3’). These nucleotides facilitate template switching by utilizing the terminal transferase activity of reverse transcriptase at the time of reaction. Dissolve the TSO in TE buffer to prepare a 100 µM stock solution and store at -80 ^{o}C.

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