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Whole-cell patch-clamping of cultured human neurons V.1

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Quyen Do[1,2,3], Dayne Beccano-Kelly[1,2], Richard Wade-Martins[1,2,3]

[1]Oxford Parkinson's Disease Centre and Department of Physiology, Anatomy and Genetics, University of Oxford, South Park Road, Oxford OX1 3QU, United Kingdom;
[2]Kavli Institute for Neuroscience Discovery, University of Oxford, Dorothy Crowfoot Hodgkin Building, South Park Road, Oxford OX1 3QU, United Kingdom;
[3]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815, USA

Cláudia C. Mendes
University of Oxford

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We use this protocol and it's working

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Abstract

This protocol describes procedures of the whole-cell patch clamping of human neurons derived from induced pluripotent stem cells and cultured in adherent monolayer.

Materials

Reagents:

- C6H11KO7 (CAS# 299-27-4)
- **Cesium Gluconate (Cs-Gluc)** (C6H11CsO7) (Hello Bio)
- **Cesium hydroxide monohydrate** (CsOH) (Sigma&Aldrich, CAS# 35103-79-8)
- EGTA (CAS# 67-42-5)
- **Guanosine 5′-triphosphate sodium salt hydrate** (Na3GTP) (Sigma&Aldrich, CAS# 36051-31-7)
- **HEPES** (H3537) (CAS# 7365-45-9)
- **Magnesium Phosphate** (MgATP) (Sigma&Aldrich, CAS# 10233-87-1-1)
- **Neurobitin® Tracer SP-1120** (Vector Labs, SKU# SP-1120-20)
- **Potassium Hydroxide (powder)**(KOH) (Sigma&Aldrich, CAS# 1310-58-3)
- **Sodium Chloride** (Sigma&Aldrich, CAS# 7647-14-5, SKU# S9888-25G)

Equipment:

- AHS-LAMP 12V/100W Halogen Lamp (Olympus, Scientifica, CAT# CLD-LAM28)
- Borosilicate glass pipettes (Premium Thin Wall Borosilicate Capillary Glass with Fillament, Model G150TF-4) (Multi Channel Systems, CAT# 640805)
- Clampfit 10.7 (Molecular Device)
- CleanBench, w/M6 tapped holes (Gimbal Piston, Scientifica, CAT# 63-9012M)
- Digidata 1550B digitiser (Molecular Device, Scientifica, CAT# DD1550A0)
- Faraday Cage 36" x 48" (900mm x 1200mm) (Scientifica, CAT# 81-333-06)
- Full Perimeter Enclosure for 36" x 48" x 2" (900mm x 1200mm x 50mm) (Scientifica, CAT# 81-321-06)
- MultiClamp 700B amplifier - a computer-controlled current & patch-clamp that comes with 2 headstages (Molecular Device, Scientifica, CAT# MultiClamp 700B)
- Plan Fluorite no-cover 60x water immersion objective (LUMPLFLN60XW) (Olympus, Scientifica, CAT# N2667800)
- PLN10X/0.25 Plan Achromat objective with 10x magnification (Olympus, Scientifica, CAT# N1215800)
- SliceScope Pro 6000 with one Control cube and one PatchPad – metric (Scientifica, CAT# SSPro-6000- 00C-P)
- Sutter P-97 Flaming Brown puller (Sutter Instrument Company, WPI, CAT# P-1000)
- U-TR30NIR Trinocular tube (Olympus, Scientifica, CAT# N1511900)
- WI-DICD Condenser (Scientifica, CAT# 38183)

Preparation of Intracellular Solution (ICS):

Made in large batches of 50 mL each on ice, aliquoted into single-use tubes, and stored in -20°C.

For current-clamp and channel current recordings:

- 140 mM C6H11KO7
- 6 mM NaCl
- 1 mM EGTA
- 4 mM MgATP
- 0.4 mM Na3GTP

- 10 mM HEPES
- 0.01% Neurobiotin (SP-1120) adjusted to 290 mOsmol/l and pH 7.3 using KOH

For post-synaptic current recordings:

- 140 mM C6H11CsO7
- 6 mM NaCl
- 1 mM EGTA
- 4 mM MgATP
- 0.4 mM Na3GTP
- 10 mM HEPES
- 0.01% Neurobiotin adjusted to 290 mOsmol/l and pH 7.3 using CsOH

Preparation of Extracellular solution (ECS):

Made fresh prior to recording, to be used within 2 weeks, and kept at 4°C.

- 2.4 mM KCl
- 167 mM NaCl
- 10 mM Glucose
- 10 mM HEPES]
- 1 mM MgCl2
- 2 mM CaCl2 adjusted to 300 mOsmol/L and pH 7.36 with NaOH

Note

For a good seal, the practice is often to make the ICS 10-20 mOsm lower than ECS. A difference greater than that will cause cells to swell.

Troubleshooting

Pulling of glass electrodes

1 Pull borosilicate glass pipettes using a Sutter P-97 Flaming Brown puller. The pulling programme is set at 470 (i.e. Ramp) Heat, 0 Pull, 150 Velocity and 500 Pressure.

Note

Pulled glass pipettes should have resistance ranging from 8-12 MΩ.

Recording Programmes

2 **Intrinsic membrane properties:**
1. Use the Intracellular Solution (ICS) for current-clamp and channel current recordings (see **Materials**).
2. Automatically report whole-cell capacitance and input resistance from Clampex membrane test in response to small (5 mV) and brief (30 ms) voltage steps.
3. Set sampling rate at 33 kHz and holding potential at -70 mV.
4. Acquire and note down resting membrane potentials (RMP) immediately upon successful break-in in current-clamp with zero current injection.

3 **Voltage-gated channels:**
1. Use the Intracellular Solution (ICS) for current-clamp and channel current recordings (see **Materials**).
2. Record simultaneously NaV and KV currents in voltage clamp.
3. Set the protocol of a series of 400 ms square voltage steps of 10 mV increments from -70 mV to +70 mV.
4. Set signal sampling and filter rate at 10 kHz and 2 kHz, respectively.
5. Pre-set leak subtraction with 4 sub-sweeps and a settling time of 250 ms.

4 **Induced action potential:**
1. Use the Intracellular Solution (ICS) for current-clamp and channel current recordings (see **Materials**).
2. Record evoked action potentials in current clamp.
3. Set the protocol of a series of 500 ms current steps in 10 pA increments from -10 pA to +130 pA.

Note

If a current injection of more than 200 pA is required to hold the cell at -70 mV, reject the recording(s).

The above Recording Programmes (steps 2, 3 and 4) can done in sequential order from the same neurons.

5 **Spontaneous excitatory (EPSC) and inhibitory postsynaptic potential (IPSC):**
1. Use the Intracellular Solution (ICS) for post-synaptic recording (see **Materials**).
2. Record spontaneous EPSC and IPSC in voltage-clamp mode at reversal potential for inward inhibitory post-synaptic current (i.e. mostly via chloride-based channel) and inward excitatory post-synaptic current (i.e. mostly via AMPA receptors) i.e. -70 mV and 0 mV, respectively.
3. Record 1-4 mins in duration with 20x gain.

Note

Each recording are performed preferentially for 2 mins. However, when cells look unhappy due to prolonged recordings, terminate the recording.

These recordings are performed on a different set of neurons due to the use of a different ICS.

Preparation of the RIG

6 Pre-warm the external solution to 27°C in a water bath.

7 Turn on controllers, digitiser, commander, camera and microscope.

8 Open HCImageLive (for live image), Clampex 10.6 (and open membrane test) and MultiClamp 700B Commander.

9 Use a 1 ml syringe and needle, suck up internal solution (thawed on ice) and replace the needle with filter and tip.

10 Inject some solution into a small tube to place the glass tubings.

Note

A similar step should be done for reverse capillary action to prevent water bubble forming at the tip.

11 Wash the recording chamber with water first, and then with ECS.

12 Adjust the position of the reference electrode towards the edge of the slope of the recording chamber to avoid direct contact with the cell layer.

13 Go to Configure and choose the right digitiser and scan. Make sure security key (as a USB drive) is inserted.

Whole-cell patch clamping measurements using Clampex

14 Place the coverslip into empty plate, rinse with some external solution.

15 Transfer the coverslip onto the recording platform.

16 Search for a healthy cell to patch under the microscope and elevate the objective along y axis.

17 Take the pulled glass pipette, inject sufficient internal solution into it, and flick it to get rid of air bubbles at the tip.

18 Secure the glass pipette into the pipette holder on the manipulator.

19 Submerge the glass pipette into the recording platform with ICS to check for intact circuit and pipette resistance.

Note

Make sure the silver rod within the electrode on the manipulator is in contact with the ICS.

20 Switch to “Bath" mode on membrane test and press play icon.

21 Dip the glass pipette into the solution at the platform.

22 Apply (and maintain) some positive pressure before dipping (with syringe) to keep small particles away from the tip of the glass tubing.

23 Lower the objective into the solution at the platform.

24 Find the tip.

25 Move the tip down, then chase by moving objective down to focus. Continue until you are close to the cell.

26 Set the amplifier to voltage-clamp and press Pipette Offset Auto.

27 Switch to slow movement for the tip and objective operator.

28 Repeat **steps 24 and 25**, in finer increments until you can see the top layer of the cell and the glass electrode tip stay directly above, but not touching, the cells.

29 Start blowing while approaching the cell, observe increase in membrane resistance.

30 As the tip makes contact the cell membrane, wait for the pipette resistance shown on the membrane test panel to reach 1 GΩ.

31 As the tip touches the cell, a very small dimple is often seen on the cell’s membrane.

32 Upon contact, release the positive pressure and the seal should be formed spontaneously or slightly suck up to create negative pressure so as to facilitate the formation of the GΩ seal.

33 Following successful formation of a tight GΩ seal, snap-suck up to rupture the cell membrane.

34 Check for good access resistance (<50 MΩ).

Note

If access resistance exceeds 50 MΩ, reject the cell. Check for access resistance again immediately following the completion of the recording, should there be significant fluctuation inn access resistance before and after the recording, reject that recording.

35 Select and run desired recording programmes as described in ***Section: Recording Programmes***.

Note

Although multiple protocols can be run within the same neurons, recording can be terminated as soon as the cell becomes unhealthy (e.g. leaky membrane, swelling) or access resistance exceeds 50 MΩ.

36 Capture brightfield images of the patched neurons for posthoc identification.

36.1 Replace ECS in the recording chamber with fresh ECS after recordings of each cell.

36.2 Patch each coverslip for no longer than 2 hours.

36.3 Transfer the patched coverslip to 12-well plate and fix with 2% PFA for 20 mins.

36.4 Keep fixed coverslips in PBS at 4°C till immunostaining.

37 Upon recording completion, switch to "Bath'" mode and very slowly and gently pull the tip and objective away from the cell.

Note

This step is to re-establish the patch-clamp configuration by slowly moving the recording pipette in small steps, alternating upward (along the Z-axis) and outward (along the X-axis) in voltage-clamp mode. Holding the cell at a depolarized will facilitate the re-sealing process.

38 Simultaneously monitor the cell capacitance and input resistance using the membrane test to visualize the loss of capacitive transients and the collapse of the current responses to a straight line, indicating the re-sealing of the cell and the establishment of an outside-out-patch at the pipette tip.

Note

Do not apply positive pressure to the recording pipette during this procedure or while detaching the cell from the pipette.

The presence of cellular debris or a membrane patch at the end of the detached tip typically indicates dislocation of the soma and will not yield complete cellular morphology.

39 After successful detachment of the pipette from the cell, retain the coverslip in the recording platform for a little while longer for even distribution of the neurobiotin along the processes.

40 Replace ECS in the recording chamber with fresh ECS after recordings of each cell.

41 Patch each coverslip for no longer than 2 hours.

42 If you intend to perform immunocytochemistry on these cells, transfer the patched coverslip to a 12-well plate and fix with 2% PFA (diluted in PBS) for 20 mins.

43 Keep fixed coverslips in PBS at 4°C till immunostaining.

Note

Fixed cells should be immunostained as soon as possible, preferably within 72 hours since fixation. We recommend performing subsequent immunocytochemistry following steps described in the **Protocol: Immunocytochemistry of cultured human Medium Spiny Neurons (MSNs)**.

Analysis

44 All analysis is performed on Clampfit 10.7.

Intrinsic membrane properties

1. Record down whole-cell capacitance and input resistance from Clampex membrane test in response to small (5 mV) and brief (30 ms) voltage steps in voltage-clamp mode.

2. Acquire resting membrane potentials (RMP) immediately upon successful break-in in current-clamp with zero current injection.

Note

While in voltage-clamp mode, If a current injection of more than 200 pA is required to hold the cell at -70 mV, reject the neurons.

EPSC and IPSC:

1. All traces are automatically low-pass Bessel 8-pole filtered.

2. Spontaneous post-synaptic events and event magnitude are automatically detected based on absolute magnitude difference from the baseline (>10 pA for EPSCs and >15 pA for IPSCs) using threshold search function on Clampfit.

3. Putative EPSC or IPSC events are excluded based on template-dependent criteria including rise time and half-width (<4 ms and <1.2 ms, respectively) and manually validated to reject false-positive events.

Ionic current densities:

1. Perform analysis of Ionic current densities on leak-subtracted traces.

2. Na_V peak currents are identified as the maximum negative current at each membrane potential within the first 50 ms.

3. Fast A-type K_V peak currents are identified as the maximum positive current at each membrane potential within the first 50 ms.
4. Slow activating K_V peak currents are identified as the average positive current at each membrane potential of the last 50 ms.
5. Current density at each membrane potential is calculated by diving the peak current to the cell capacitance.

Induced Action Potential:
1. At each current injection, an action potential is determined as a significant upward deflection of at least 20 mV that overshoot 0.
2. Rheobase current is determined as the minimal current amplitude that results in the depolarization of the cell membranes and generation of an action potential.