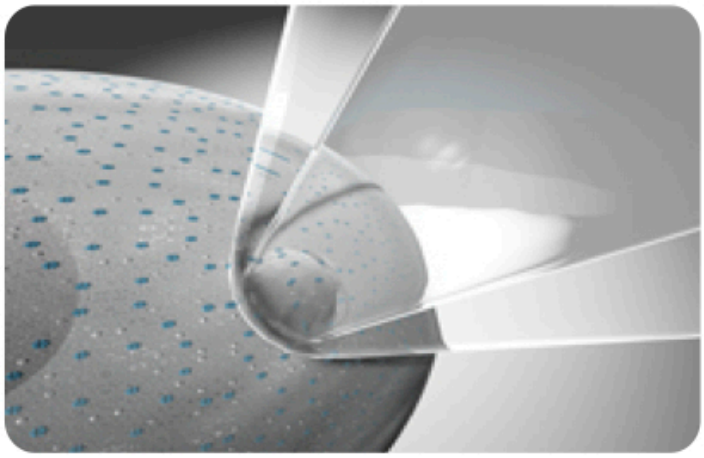
Experts' Protocol Series

Whole-cell Patch-clamp Recordings for Characterizing Neuronal Electrical Properties



Application Protocol

Kindly Provided by the Ole Paulsen Lab at the University of Cambridge

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I. INTRODUCTION

Patch-clamp is the gold standard technique for high-fidelity analysis of the electrical properties and functional connectivity of neurons. Several patch clamp configurations can be used depending on the research interests, but in all cases, electrophysiological recordings are produced using a glass micropipette in contact with a patch of the neuron's membrane. In the *cell-attached* mode, the membrane patch is left intact allowing the recording of ion channels within the patch as well as action potentials. Applying a pore-forming agent, such as amphotericin, in the pipette results in a *perforated patch,* which establishes electrical continuity whilst preventing the dialysis of intracellular proteins. However, the most commonly used patch-clamp mode is *the whole-cell* mode where the membrane patch is disrupted by briefly applying strong suction to establish electrical and molecular access to the intracellular space. This has two main configurations: the voltage-clamp mode, in which the voltage is held constant allowing the study of ionic currents, and the current-clamp mode, in which the current is controlled enabling the study of changes in membrane potential.

Several books have been written describing this technique in detail. Described here, is a simplified protocol of the *whole-cell* patch clamp technique, for use in neuronal cultures. This protocol has been used to generate the results described below. The solutions and voltage and current steps used are specific for these recordings and can be modified according to the scientist's requirements.

II. MATERIALS REQUIRED

Solutions

Artificial cerebrospinal fluid (aCSF)

Composition: 126 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 26.4 mM $NaHCO_3$ and 10 mM glucose.

Preparation: Make up 1 L 10X stock solution containing only $NaHCO_3$, another 1 L 10X stock solution containing the rest of the components and store for up to one week at 4 °C. Before recording, prepare 1X solution; adjust osmolarity to 290 mOsm (+/- 10 mOsm) and bubble with carbogen (95% O_2 – 5% CO_2).

IMPORTANT: This aCSF recipe uses a bicarbonate buffer; alternatively, HEPES (10-15 mM) can be used to buffer the pH of the solution. If a HEPES buffered solution is used, $NaHCO_3$ and CO_2 bubbling are not required.

Intracellular solution

Composition: 115 mM K-Gluconate, 4 mM NaCl, 0.3 mM GTP-NaCl, 2 mM ATP-Mg, 40 mM.

Preparation: Make up 1X solution; adjust the pH to 7.2 with KOH and the osmolarity at 270 mOsm L^{-1} (+/- 10 mOsm L^{-1}). Aliquot the solution and store at -20 °C. Before recording, thaw an aliquot and filter it using a standard 0.2 µm filter. Use a 1 mL syringe with a microloader tip to load the recording pipettes. If analysis of cellular morphology post hoc is required, include an intracellular dye or label in the internal solution (e.g. biocytin, neurobiotin, etc) and reduce the concentration of K-Gluconate correspondingly.

Electrophysiology equipment setup

- Anti-vibration table (optionally with a Faraday cage)
- Microscope
- Micromanipulator
- Borosilicate glass capillary holder
- Pressure control system
- Headstage
- Amplifier
- Data interface
- Computer
- Recording software
- Camera
- Display screen
- Perfusion pump
- Carbogen (95% O_2 and 5% CO_2) input

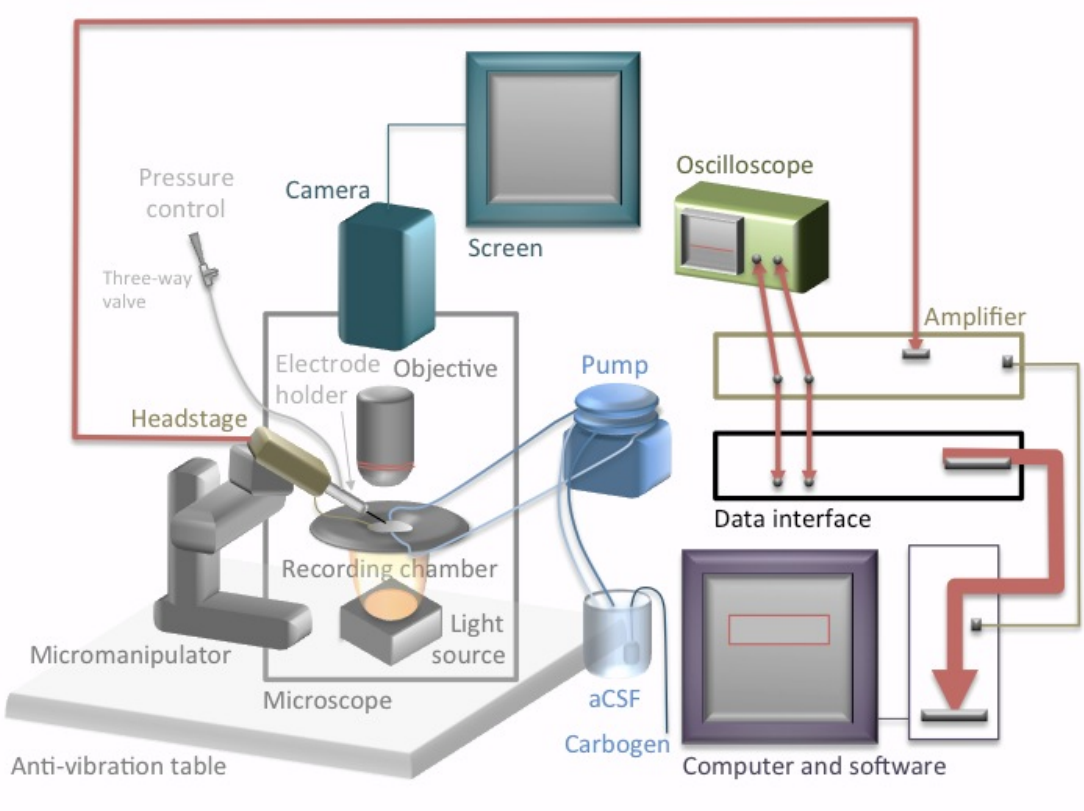

Figure 1. Whole-cell patch clamp set up. The red arrows show the direction of the electrical signal recorded.

Consumables

- Borosilicate glass capillaries: 0.68 mm inner diameter (ID) and 1.20 mm outer diameter (OD) with filament
- 0.2 µm pore filters
- 20 µL Micro-loader tips
- Three-way valve
- Tubes for perfusion system, pressure system and carbogen system.
- Carbogen (95% O_2 and 5% CO_2) tank with regulator
- Air stone to deliver carbogen to the aCSF

Other equipment required

- Glass capillary puller
- Osmometer (optional)

III. WHOLE-CELL PATCH CLAMP PROCEDURE

Preparation

1. Plate the neurons a few days prior to recording onto coverslips.
2. Turn on all the equipment and set the pump to perfuse aCSF through the recording chamber (a commonly used speed for whole-cell patch clamp in cultures is 1.5 mL per minute).

   IMPORTANT: a perfusion speed over 2 mL per minute might lead to movements of the recording pipette and lifting of the cells from the coverslip.

3. Place the coverslip with cells in the recording chamber with the cells facing up.

4. Use a glass capillary puller to make two recording pipettes from a borosilicate glass capillary with a resistance of between 3 and 7 MΩ when filled with K-Gluconate based internal solution.

   IMPORTANT: 5 MΩ is the standard resistance used for most recordings. Lower resistance pipettes (of 3-4 MΩ) will give a lower series resistance and thus, will be better for voltage-clamp recordings. However, the tip of the glass pipette will be larger than for higher resistance pipettes and, as a consequence, the seal formation will be challenging and the seal will be more difficult to keep stable over time. Higher resistance glass pipettes (of 6-7 MΩ, with a thinner tip) are easier to form a seal with and can be more suitable for prolonged current-clamp recordings; however, they are slightly more difficult to break through the membrane into whole-cell mode and will give a higher series resistance, sometimes too high for reliable voltage-clamp recordings.

5. Fill a 1 mL syringe with 200 µL of intracellular solution, connect a 0.2 µm pore filter to the syringe and attach a micro-loader tip to the filter. Alternatively, filter 200 µL of intracellular solution with a 0.2 µm pore filter, fill a 1 mL syringe with the filtered solution and attach a micro-loader tip to the syringe barrel.

Main procedure

1. Find a cell to patch. Do not move the microscope stage for the rest of the procedure. If using an upright microscope, move the objective outside the bath.

2. Use the syringe linked to the filter and micro-loader tip to fill halfway a borosilicate pipette with intracellular solution. Tap the pipette a few times to eliminate any air bubbles that might be present in the tip of the pipette.

3. Place the glass pipette in the pipette holder. Place the pipette tip in the bath and focus the tip.

4. Once the pipette is in the bath, apply very light positive pressure through the pressure control system and hold the pressure in the pipette by closing the three-way valve.

5. Approach the coverslip by moving the micromanipulator and monitoring the pipette height on the screen. Always focus at or below the tip of the glass pipette.

6. Stop moving the glass pipette just before approaching the cell layer on the coverslip, making sure that the cell bodies are not in focus at this stage. Change the settings of the micromanipulator to smooth and slow motion.

7. Set the amplifier to voltage-clamp and correct the pipette offset so the currents measured at that point are considered as 0 pA. Apply a seal test (a 10 mV test pulse at 100 Hz) through the recording electrode.

   IMPORTANT: The oscilloscope should show the square current response to the seal test. Following Ohm's law (current (I)= voltage (E) / resistance(R)), the amplitude of the response will depend on the resistance of the pipette.

8. Approach the cell body moving the glass pipette along its long axis until the tip touches the cell and a very small dimple is seen on the cell's membrane.

   IMPORTANT: Do not patch the nucleus of the cell. Approach the cell away from the nucleus.

9. Release the positive pressure to obtain a GΩ seal. The pressure should be so light that a seal could form spontaneously only by approaching the cell.

   IMPORTANT: A GΩ seal is characterised by a resistance that reaches at least 1 GΩ. The current response to the seal test should be very small and if the scale of the oscilloscope is kept unchanged, the response should appear almost flat.

10. Once a GΩ seal has been formed, change the voltage clamp to a negative voltage close to the expected cell resting potential (- 60 to -70 mV) and correct for fast capacitance.

11. To break through the membrane, apply light and short suction pulses using a syringe (or by mouth only if it is safe to do so). If the membrane does not break, try applying stronger suction or apply brief electrical pulses through the pipette if the patch clamp amplifier has a 'zap' function.

    IMPORTANT: Since the membrane of the cell acts as a capacitor, when the glass pipette has gained access to the intracellular space, the current response to the seal test should show an exponential decay.

12. Acquire and analyse recordings using the appropriate software.

    a) For current clamp experiments, change to current-clamp mode, read the resting membrane potential at zero current, and adjust the membrane potential to -60 to -70 mV by applying current if required. To analyse the passive membrane properties and the spiking properties of the cells, apply alternate steps of negative and positive current. To confirm the spikes seen are sodium spikes, tetrodotoxin (TTX) can be used to block voltage-dependent sodium channels.

    b) For voltage-clamp recordings, continue in voltage-clamp mode and remove the seal test. Hold the cell at -70 mV to record spontaneous excitatory postsynaptic currents (EPSCs) and at 0 mV to record spontaneous inhibitory postsynaptic currents (IPSCs). Blockers of AMPA receptors, NMDA receptors or GABA receptors can be applied to better isolate the currents of interest or to confirm the presence of a specific current.

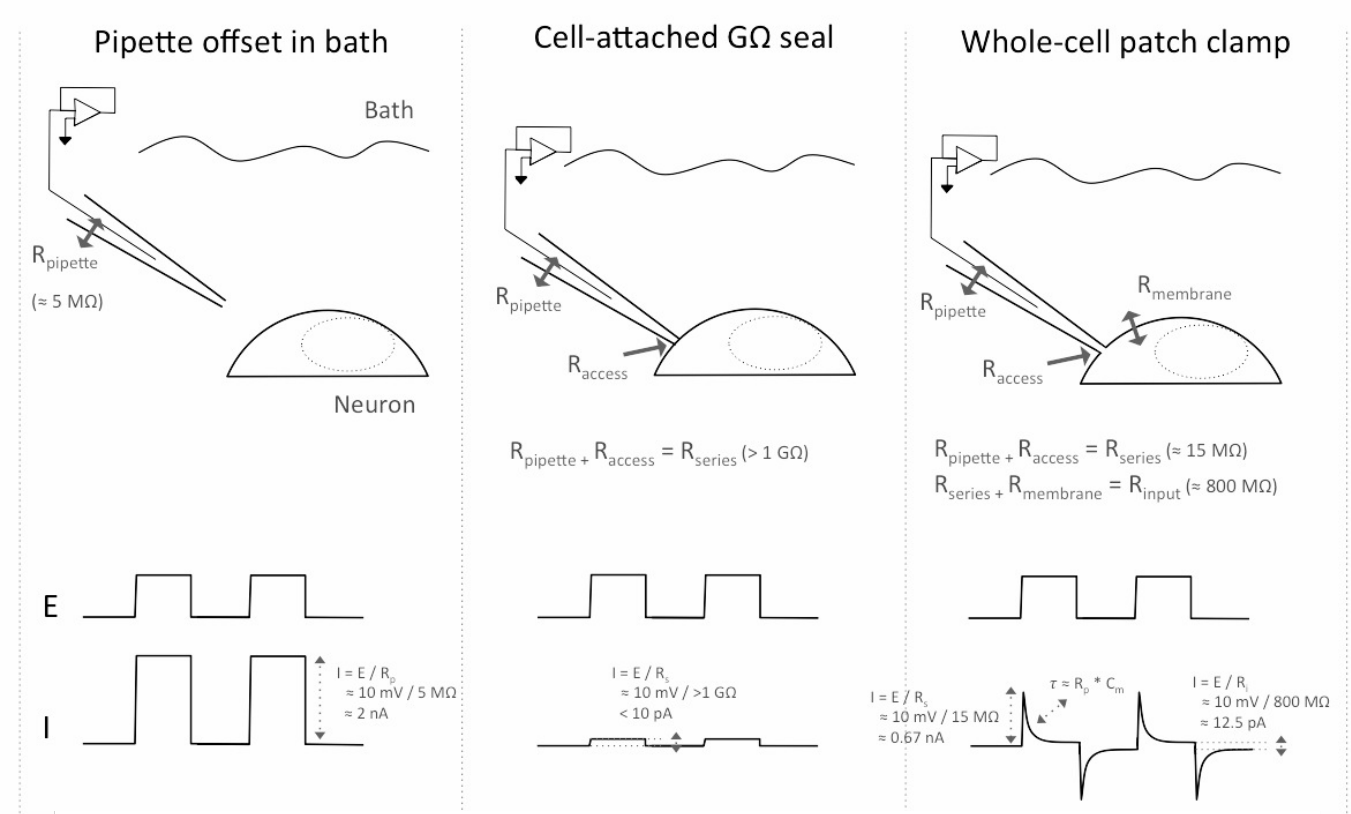

Figure 2. Three main steps on whole-cell patch clamp procedure. The readout on the oscilloscope is shown for all three steps.

IV. ANTICIPATED RESULTS

Whole-cell patch clamp can be used to characterize the maturation of neuronal cultures, both at the level of individual cells and at the network's connectivity level. As neurons derived from Axol™ hNPCs mature over time, the number of cells spiking increased up to 100% of the total number of neurons recorded at one month after plating (**Figure 3A**). The maturation stage of neurons is reflected by their spiking profile. Over the course of maturation, neurons express more voltage-dependent Na^+ channels causing higher amplitude action potentials and more K^+ channels producing a reduction in the spike's width (**Figure 3B and C**). Along with a modification in the spiking profile, synaptic connections start to appear after one month in culture (**Figure 3E**). The enrichment on excitatory and inhibitory connections to cells after 45 days in culture suggests a fully mature neuronal network (**Figure 3F**).

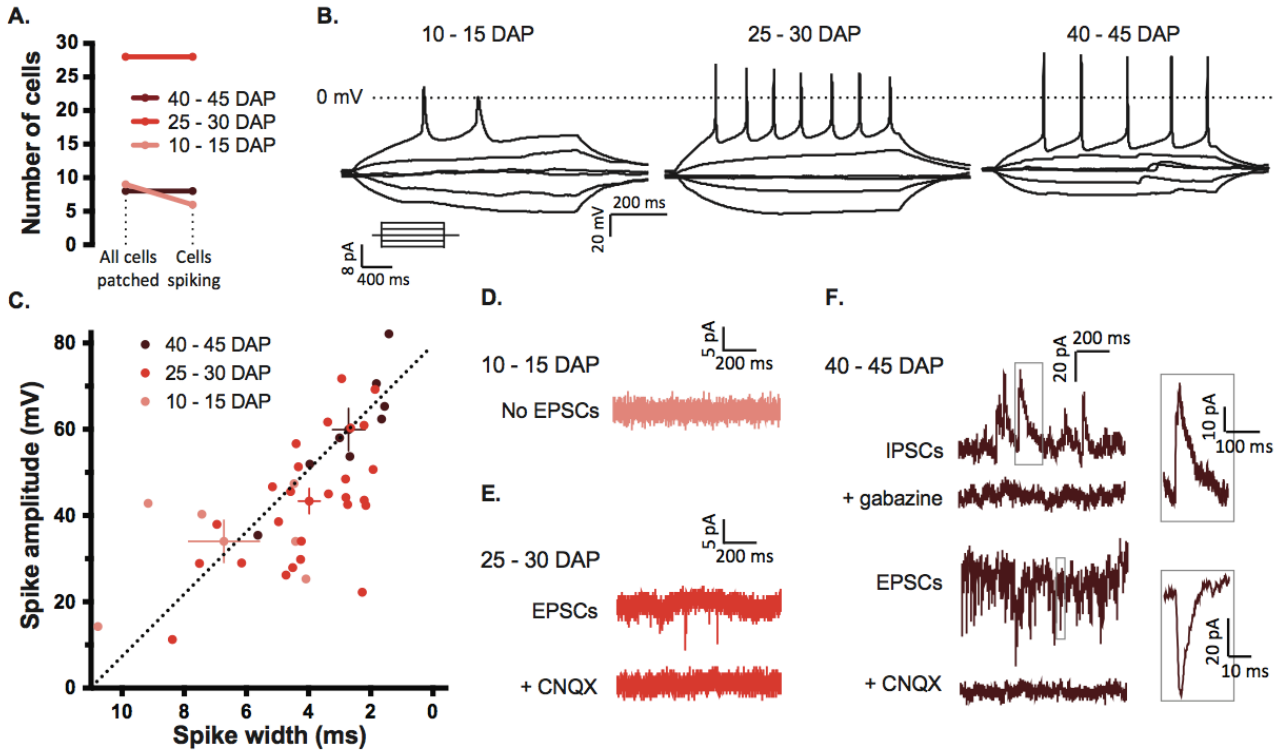

Figure 3. Electrophysiological characterisation of neurons derived from Axol™ hNPCs. **A.** Number of cells recorded that showed evoked action potentials compared to the number of total cells recorded. Three different developmental stages were analysed: 10 to 15 days after plating (DAP) in coverslips, 25 to 30 DAP and 40 to 45 DAP. **B.** Representative traces of evoked action potentials. **C.** Developmental profile of the spike properties of neurons derived from hNPCs. **D.** Voltage clamp recording at -70 mV from hNPCs at 10 to 15 DAP. No synaptic currents were detected. **E.** 25 to 30 DAP, some synaptic currents were observed. These currents were excitatory postsynaptic currents (EPSCs) and were blocked by CNQX (10 µM), an AMPA and kainate receptor blocker. **F.** Fully mature neurons at 40 to 45 days post-plating showed both EPSCs and inhibitory postsynaptic currents (IPSCs), which could be blocked using a $GABA_A$ receptor blocker (gabazine, 2 µM). Inhibitory postsynaptic currents (IPSCs) were recorded at 0 mV.

V. TROUBLESHOOTING

Problem	Possible Cause	Solution
The pipette offset does not set the current and voltage to 0.	The reference electrode is not in the bath or the electrode wire in the pipette holder might not be connected.	Place the reference silver pellet in the bath and connect it to the headstage. Check the electrode wire.
The response drifts.	The electrode is not chloridated.	Put the end of the silver wire used as the recording electrode in bleach for 30 minutes and wash thoroughly or dip it in a 300 mM NaCl solution and pass current from a 5 V source through. A dark layer of AgCl should appear.
The resistance of the pipette increases >15 MΩ when pressure is applied.	Dust is clogging the tip of the pipette.	Change the pipette.
The resistance of the pipette is > 50 MΩ with visible capacitance.	A bubble is clogging the tip of the pipette.	Try applying high pressure to make the bubble leave the pipette. Otherwise take the pipette out and give it a few taps.
The resistance of the pipette is < 1 MΩ.	The pipette tip is broken.	Change the pipette.
Difficulties in making a GΩ seal.	Not enough negative pressure applied.	Apply some more negative pressure; close the three-way valve and wait a few minutes. Setting the voltage at -60 to -70 mV can help.
	Cells are unhealthy.	Make sure the cells have been constantly oxygenated. Check the pH and osmolarity of the aCSF and internal solution.
	The pipette resistance is under 4 MΩ or over 8 MΩ.	Change the resistance of your pipette to 6-7 MΩ.

	The pressure system is not working.	Check the pressure line and the three-way valve for leaks.
	The pipette is dirty	Check the resistance of the pipette. Change the pipette if it is clogged.
The cell body is aspirated inside the pipette when trying to make the seal.	The resistance of the pipette is too low.	Use a higher resistance pipette.
	Too much positive pressure applied causing the aspiration of part of the soma when the pressure is released.	Use very light to none positive pressure.
Difficulties to break through the membrane.	The pipette resistance is over 8 MΩ.	Lower the resistance of the pipette.
	The pressure system is not working	Check the pressure line and the three-way valve for leaks.
	Mouth suction is not enough.	Use the “zap” function in the amplifier to provide short electric pulses through the pipette to create small holes on the membrane to help breaking through.
Once the membrane has been broken, the access resistance is too high.	The membrane is not completely broken.	Keep applying short soft suction pulses by mouth. The access resistance should be < 25 MΩ.
Difficulties maintaining the patch.	The resistance of the pipette is too low and the seal is lost.	Use a higher resistance pipette.
	The pipette is moving.	Check the anti-vibration table, the micromanipulator stability and the microscope stage.
	The coverslip is moving.	Check the flow of aCSF, make sure it is between 1 to 1.5 mL per minute. Make sure there are no bubbles in the input of aCSF to the bath.

	The culture is unhealthy.	Make sure the cells have been constantly oxygenated. Check the pH and osmolarity of the aCSF and internal solution.
	Patching too close to the nucleus.	Select the cells in which the nucleus can be visualised but do not patch cells that look swollen. Approach the cell opposite to the nucleus.
The input resistance is increasing over time.	The patch is resealing.	Try to break through again very smoothly.
The input resistance is decreasing over time.	The GΩ seal is lost or the cell is leaking through opening ion channels (e.g. K).	Depending on the experiments done, cells might be discarded if the resistance changes under a certain threshold.

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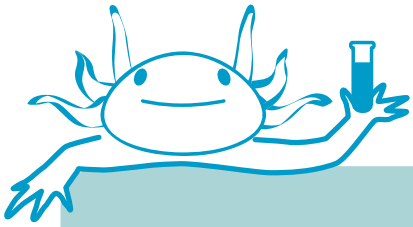

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