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Nuclear Patch-Clamp Electrophysiology of Ca²⁺ Channels

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Abstract

Patch-clamping the outer or inner nuclear membrane of isolated nuclei is very similar to patch-clamping the plasma membrane of isolated cells. This protocol describes in detail all the steps required to successfully obtain nuclear membrane patches, in various configurations, from both the outer and inner nuclear membranes of isolated nuclei.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Bath solution appropriate for channel activity:

- Bath solution for inositol 1,4,5-trisphosphate receptors (InsP₃R) <R>
- Bath solution for ryanodine receptors (RyR) <R>

Cell and tissue adhesive (e.g., Cell-Tak; BD Biosciences 354240)

Cell homogenate containing nuclei in NIS-O (with the outer nuclear membranes intact) or in NIS-I (with the outer nuclear membranes removed)

See Isolating Nuclei from Cultured Cells for Patch-Clamp Electrophysiology of Intracellular Ca²⁺ Channels

(Mak et al. 2013a).

Ethanol (99% purity)

Patch pipette solution appropriate for channel activity:

- Patch pipette solution for InsP₃R <R>
- Patch pipette solution for RyR <R>

Equipment

Standard equipment for performing regular plasma membrane patch-clamp experiments is described in Penner (1995). Further special requirements for nuclear patch-clamp electrophysiology experiments are listed below.

Coverslips (glass)

Inverted microscope (with long-distance 20× air and 60× oil-immersion objectives)

Patch-clamp amplifier (with headstage mounted on the micromanipulator so that the micropipette sustains an angle at least 45° to the horizontal)

Patch-clamp micropipette (with tip resistance of ~10–25 MΩ in 140 mM KCl solution)

Perfusion system (e.g., VC-6M mini-valve control system with SF-77B perfusion fast-step system from Warner Instruments)

This is a local fast-switching perfusion system with flow controls so rapid perfusion solution exchange can be performed.

Pressure gauge (DALE20 pneumatic transducer tester from Ultramedic Ltd.)

Three-axis micromanipulator (e.g., the MP-285 programmable motorized micromanipulator from Sutter Instrument)

This instrument has slow-motion capability (a steady speed of ~3 μm/sec) and can be used to excise nuclear membrane patches in various configurations.

METHOD

Nuclear patch-clamping can be performed with various nuclear membrane patch configurations depending on the experiment (see Fig. 2 in **Patch-Clamp Electrophysiology of Intracellular Ca²⁺ Channels**

[Mak et al. 2013b]).

- On-nucleus configuration (Steps 1–4): The patch-clamp micropipette makes contact with the outer nuclear membrane of an isolated nucleus. In this configuration, the cytoplasmic side of the isolated membrane patch (and of any InsP₃R channel in that patch) faces the pipette solution.
- Luminal-side-out (lum-out) configuration (Step 5): The patch is excised and in this configuration the luminal aspect of membrane (and any InsP₃R channel) is exposed to the bath or perfused solution.
- Cytoplasmic-side-out (cyto-out) configuration (Steps 6–12): This configuration is achieved by breaking through to the perinuclear region with brief high voltage pulses followed by slow excision. The cytoplasmic side of the membrane (and any InsP₃R channel) is now exposed to the bath or perfused solution.
- *Patching the inner nuclear membrane (Steps 13–14): This is achieved by using nuclei from which the outer nuclear membrane has been removed (see **Isolating Nuclei from Cultured Cells for Patch-Clamp Electro-physiology of Intracellular Ca²⁺ Channels** [Mak et al. 2013a]).*

On-Nucleus Configuration

- 1 Fill the experimental chamber on the stage of the microscope with appropriate bath solution (depending on the channel under investigation). Add 40–60 μL of freshly prepared cell homogenate in NIS-O (containing nuclei with outer nuclear membrane intact) per mL of bath solution. Let the nuclei settle to the bottom for 5–10 min before performing patch-clamp experiments.

The nuclei can only be used for 30–60 min under these conditions at room temperature before the success rate of observing InsP_3R channel activity starts to decline. After this time, refresh the experimental chamber and use a new aliquot of homogenate.

- 2 Focus the microscope at the bottom of the experimental chamber and scan the chamber until you find a conveniently placed “good” nucleus with outer membrane intact (see Fig. 1 in **Isolating Nuclei from Cultured Cells for Patch-Clamp Electrophysiology of Intracellular Ca^{2+} Channels** [Mak et al. 2013a]).
- 3 Lower the patch-clamp micropipette filled with the appropriate pipette solution until it comes into view above the selected nucleus, constantly applying 4–10 mmHg positive pressure to prevent cell debris from contaminating the tip of the pipette. Carefully advance the pipette toward the chosen nucleus, aiming for a clean area close to the edge of the nucleus (a perfectly round and smooth edge corresponds to a small area that is free of visible debris; see Fig. 1 in **Isolating Nuclei from Cultured Cells for Patch-Clamp Electrophysiology of Intracellular Ca^{2+} Channels** [Mak et al. 2013a]).
- 4 When the pipette tip is visibly observed to touch the nucleus (usually this contact does not change the pipette resistance, unlike with plasma-membrane patching), gradually decrease the applied pressure until 4–10 mmHg suction is sustained to obtain a giga-ohm seal. Remove the suction as soon as a giga-ohm seal is achieved. (This usually takes a few seconds, but sometimes a seal improves gradually over tens of seconds.) To maximize the duration of the recorded channel activity, start current recordings as soon as the seal resistance exceeds 100–200 $\text{M}\Omega$ (depending on the gain settings on the amplifier).

With the exception of Sf9 and Xenopus oocyte nuclei, it is advisable to start recording at a constant negative applied potential (-40 mV $V_{\text{app}} - 0\text{ mV}$, with respect to the bath electrode), as this usually speeds up the seal formation.

See Troubleshooting.

Luminal-Side-Out (Lum-Out) Configuration

- 5 After the on-nucleus configuration is stabilized and channel activity is detected, withdraw the pipette to excise the isolated patch of outer nuclear membrane, thereby exposing the luminal aspect of the channel to the bath or the locally perfused solution and producing the lum-out configuration.

See Troubleshooting.

The membrane patch observed in this configuration is essentially the same one as in the on-nucleus configuration. Therefore, if no active InsP_3R channel is detected in the membrane patch in the on-nucleus configuration, there is no reason to proceed to acquire the lum-out configuration.

Cytoplasmic-Side-Out (Cyto-Out) Configuration

A prerequisite for obtaining the cyto-out configuration is tight adhesion of isolated nuclei to the glass bottom of the experimental chamber, a situation that does not occur with nuclei from most cell types. To promote tight attachment, it is usually necessary to place glass coverslips coated with cell and tissue adhesive at the bottom of the experimental chamber.

- 6 Place 1 μL of cell and tissue adhesive on one side of a coverslip and spread it evenly over the surface. Let the coverslip dry with the adhesive-coated side facing up for 1–2 h at 4°C.
- 7 Immerse the coverslip in ethanol (99% purity) for 5 min. Rinse it several times in distilled water and let it dry with the adhesive-coated side facing up at 4°C. Coated coverslips can be stored for at least 1 wk at 4°C.
- 8 Place the coated coverslip on the bottom of the experimental chamber. For some specially designed experimental chambers, the coated coverslip can be used as the bottom of the chamber. Make sure that the adhesive-coated side faces upward.
- 9 Obtain a nuclear membrane patch in the on-nucleus configuration (see Steps 1–4).
- 10 After a stable on-nucleus configuration is established, disrupt the isolated nuclear membrane patch with brief voltage pulses (generally available with the “zap” function of most patch-clamp amplifiers). If variable voltage pulses are available, start with the one with the shortest duration or lowest intensity and gradually increase the pulse strength until the seal resistance is stably reduced to $<100\text{ M}\Omega$. Apply a concomitant 10–30 mmHg suction to help maintain this intermediate configuration in which the pipette interior is topologically continuous with the perinuclear region of the patched nucleus.

See Troubleshooting.

- 11 Release the suction. At the same time start lifting the patch-clamp micropipette up at the slowest possible rate. Monitor the seal resistance (R_s) continuously as the pipette is lifted away from the nucleus.

In a successful attempt, the R_s should gradually increase until an abrupt increase toward $\sim 1\text{ G}\Omega$ is observed. This corresponds to the pipette tip being fully detached from the patched nucleus, with the nuclear membrane “resealing” at the tip of the pipette. Most of the time the nuclear membrane reseals in the cyto-out configuration. In very few instances, however, the membrane can reseal in the opposite, lum-out configuration. To distinguish between these two possibilities, an activating ($10\text{ }\mu\text{M}$) concentration of InsP_3 should be included in the pipette solution. Absence of channel activity in the resealed patch indicates that the cyto-out configuration is achieved.

See Troubleshooting.

- 12 Once a stable cyto-out patch is established, reposition the pipette so that rapid perfusion solution exchange at the pipette tip can be performed using a local perfusion system.

The rate of achieving nuclear membrane resealing depends mainly on the cell type used. The only experimental parameter that significantly impacts the success rate is the rate at which the patch-clamp micropi-

ette is lifted from the patched nucleus: The slower the pipette is lifted, the higher the success rate.

The cyto-out membrane patch formed at the tip of the pipette after resealing is different from the patch observed in the on-nucleus configuration. Thus, obtaining a cyto-out patch should be attempted even if no active channel is detected in the on-nucleus configuration.

Patching the Inner Nuclear Membrane

- 13 Perform Steps 1–4 using freshly prepared cell homogenate in NIS-I (containing nuclei with the outer nuclear membrane removed) to achieve the on-nucleus configuration with a nucleus with the inner nuclear membrane exposed.

Because the outer nuclear membrane has been removed, the orientation of the membrane is opposite to that in on-nucleus configuration. InsP₃R or RyR channels isolated this way are activated only by adding appropriate cytoplasmic ligands (InsP₃ for InsP₃R and caffeine for RyR) into the bath solution.

- 14 To achieve the nucleoplasmic-side-out (nucleo-out) configuration, excise the isolated membrane patch from the on-nucleus configuration. This configuration provides better access to the nucleoplasmic aspect of the membrane patch for rapid perfusion experiments.

TROUBLESHOOTING

Problem (Step 4): Good quality seals cannot be obtained.

Solution: Make sure that there is no mechanical drift of the patch-clamp micropipette tip. This can be caused by either a faulty micromanipulator (fix it or have it serviced) or a worn O-ring in the pipette holder (replace it). The heating filament of the patch-clamp micropipette puller, if overused, can contaminate the tip of the glass with evaporated metal and prevent a good seal. Replace it if necessary.

Problem (Step 5): It is difficult to attain the lum-out configuration.

Solution: The major reason for this problem is that nuclei do not attach strongly to the glass bottom of the experimental chamber and so, when the pipette is gently withdrawn from the patched nucleus, the whole nucleus is lifted up with the pipette. In this case, withdraw the pipette from the nucleus quickly. If this fails and the nucleus remains attached to the pipette tip, try gently pressing the pipette tip against the bottom of the dish and then gently withdrawing the pipette from the nucleus. Alternatively, lift the pipette with the attached nucleus up until it is at a safe distance from the bottom of the experimental chamber and then gently tap the patch-clamp headstage to remove the nucleus. The success rate with this procedure depends on the particulars of the micromanipulator that is used. With the techniques described, we can achieve the lum-out configuration with a success rate of 40%–80%, depending on the cell type used. Failure of the above-mentioned maneuvers may result in complete loss of the giga-ohm seal so seal resistance decreases significantly, or formation of a vesicle at the tip of the pipette so the channel activity is lost without a decrease in seal resistance. In the latter situation, attempts to get the proper lum-out configuration by transiently removing the pipette from the bath have never succeeded.

Problem (Step 10): Disrupting the nuclear membrane patch consistently fails, even with zapping and application of suction at the same time.

Solution: Reduce the suction applied during on-nucleus patch formation (Step 4).

Problem (Step 11): Low seal resistance for excised cyto-out nuclear membrane patches is observed.

Solution: Although resealing usually occurs very quickly, sometimes it can take longer. Wait some time (~5–20 sec) to see if the seal resistance improves before positioning the pipette for rapid perfusion experiments.

DISCUSSION

Nuclear patch-clamping is very similar to patch-clamping the plasma membrane of cells, but there are challenges and limitations that are unique to this method. To mimic the physiologically low free $[Ca^{2+}]$ and $[Mg^{2+}]$ in the cytosol, solutions used in nuclear patch-clamping contain relatively low amounts of divalent cations. Although there is no detectable decrease in the success rate of obtaining giga-ohm seals relative to plasma membrane patch-clamping in general, the quality (seal resistance) and stability of the giga-ohm seal obtained in the on-nucleus configuration are lower than those for on-cell plasma membrane patches since divalent cations have beneficial effects on giga-ohm seals (Priel et al. 2007). This leads to higher background current noise in nuclear membrane patches. However, this does not pose a serious problem because of the large conductances of the intracellular Ca^{2+} channels. In general, in comparison to intact cells grown on coverslips, isolated nuclei do not attach well to the bottom of the experimental chamber. This problem, together with the lack of a cytoskeleton to support the nuclear envelope, makes it difficult to obtain excised nuclear patches, especially in the cyto-out configuration.

RELATED INFORMATION

For a discussion of the application of nuclear patch-clamp experiments to the study of $InsP_3R$ and RyR channels in the outer and inner nuclear membrane and their role in $[Ca^{2+}]_i$ signaling, see **Patch-Clamp Electrophysiology of Intracellular Ca^{2+} Channels** (Mak et al. 2013b).

RECIPES

Bath Solution for $InsP_3R$

Reagent	Final concentration
KCl	140 mM
HEPES	10 mM
$CaCl_2$	0.06 mM
BAPTA (1,2-bis[<i>o</i> -aminophenoxy]ethane- <i>N,N,N',N'</i> -tetraacetic acid)	0.5 mM

Adjust the pH of the solution to 7.3 with 1 M KOH.

Bath Solution for RyR

Reagent	Final concentration
KCl	250 mM
HEPES	25 mM
$CaCl_2$	0.18 mM

Reagent	Final concentration
BAPTA (1,2-bis[<i>o</i> -aminophenoxy]ethane- <i>N,N,N',N'</i> -tetraacetic acid)	0.5 mM

Adjust the pH of the solution to 7.3 with 1 M KOH.

Patch Pipette Solution for InsP₃R

Reagent	Final concentration
KCl	140 mM
HEPES	10 mM
Na ₂ ATP	0.5 mM
CaCl ₂	* mM
Ca ²⁺ chelator	** 0.5 mM

Adjust the pH of the solution to 7.3 with 1 M KOH.

* Use the online software Webmaxc Standard (<http://www.stanford.edu/~cpatton/web-maxcS.htm>) to estimate the amount of CaCl₂ that should be used to obtain the desirable free Ca²⁺ concentration ([Ca²⁺]_f) in the final solution. Confirm the actual [Ca²⁺]_f (<100 μM) in final solutions by Ca²⁺-sensitive dye fluorimetry. Do not use Ca²⁺ chelator for solutions with [Ca²⁺]_f >100 μM. Calculate [Ca²⁺]_f in these solutions by using the appropriate Ca²⁺ activity coefficients (Butler 1968; Vais et al. 2010).

** Use BAPTA (1,2-bis[*o*-aminophenoxy]ethane-*N,N,N',N'*-tetraacetic acid) when 20 nM < [Ca²⁺]_f ≤ 600 nM. Use 5,5'-dibromo BAPTA when 600 nM < [Ca²⁺]_f ≤ 4 μM. Use HEDTA (hydroxyethylethylenediaminetriacetic acid) when 4 μM < [Ca²⁺]_f ≤ 20 μM. Use NTA (nitrilotriacetic acid) when 20 μM < [Ca²⁺]_f ≤ 100 μM. ATP contributes to Ca²⁺ buffering in solutions with [Ca²⁺]_f >30 μM (Bers et al. 2010).

Patch Pipette Solution for RyR

Reagent	Final concentration
KCl	250 mM
HEPES	25 mM
Na ₂ ATP	0.5 mM
CaCl ₂	* mM
Ca ²⁺ chelator	** 0.5 mM

Adjust the pH of the solution to 7.3 with 1 M KOH.

* Use the online software Webmaxc Standard (<http://www.stanford.edu/~cpatton/web-maxcS.htm>) to estimate the amount of CaCl₂ that should be used to obtain the desirable free Ca²⁺ concentration ([Ca²⁺]_f) in the final solution. Confirm the actual [Ca²⁺]_f (<100 μM) in final solutions by Ca²⁺-sensitive dye fluorimetry. Do not use Ca²⁺ chelator for solutions with [Ca²⁺]_f >100 μM. Calculate [Ca²⁺]_f in these solutions by using the appropriate Ca²⁺ activity coefficients (Butler 1968; Vais et al. 2010).

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References

Bers DM, Patton CW, Nuccitelli R. A practical guide to the preparation of Ca²⁺ buffers. *Methods Cell Biol.* 2010; 99:1–26. [PubMed: 21035681]

- Butler JN. The thermodynamic activity of calcium ion in sodium chloride-calcium chloride electrolytes. *Biophys J.* 1968; 8:1426–1433. [PubMed: 5713452]
- Mak D-OD, Vais H, Cheung K-H, Foscett JK. Isolating nuclei from cultured cells for patch-clamp electrophysiology of intracellular Ca^{2+} channels. *Cold Spring Harb Protoc.* 2013a10.1101/pdb.prot073056
- Mak D-OD, Vais H, Cheung K-H, Foscett JK. Patch-clamp electro-physiology of intracellular Ca^{2+} channels. *Cold Spring Harb Protoc.* 2013b10.1101/pdb.top066217
- Penner, R. A practical guide to patch-clamping. In: Sakmann, B.; Neher, E., editors. *Single-Channel Recording*. Plenum; New York: 1995. p. 3-30.
- Priel A, Gil Z, Moy VT, Magleby KL, Silberberg SD. Ionic requirements for membrane–glass adhesion and giga seal formation in patch-clamp recording. *Biophys J.* 2007; 92:3893–3900. [PubMed: 17369408]
- Vais H, Foscett JK, Mak D-OD. Unitary Ca^{2+} current through recombinant type 3 InsP_3 receptor channels under physiological ionic conditions. *J Gen Physiol.* 2010; 136:687–700. [PubMed: 21078871]