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TITLE:

An Efficient Electroporation Method to Study Primary Cilium-dependent Signaling Pathways in the Granule Cell Precursor

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KEYWORDS: Primary cilia, cerebellar granule precursor, *in vitro* electroporation, Hedgehog signaling pathway, primary GCP culture, Smoothened.

SUMMARY:

Here, we present a reproducible *in vitro* electroporation protocol for genetic manipulation of primary cerebellar granule cell precursors (GCPs) that is cost-effective, efficient, and viable. Moreover, this protocol also demonstrates a straightforward method for the molecular study of primary cilium-dependent Hedgehog signaling pathways in primary GCP cells.

ABSTRACT:

The primary cilium is a critical signaling organelle found on nearly every cell that transduces Hedgehog (Hh) signaling stimuli from the cell surface. In the granule cell precursor (GCP), the primary cilium acts as a pivotal signaling center that orchestrates precursor cell proliferation by modulating the Hh signaling pathway. The investigation of primary cilium-dependent Hh signaling machinery is facilitated by *in vitro* genetic manipulation of the pathway components to visualize their dynamic localization to the primary cilium. However, transfection of transgenes in the primary cultures of GCPs using the currently known electroporation methods is generally costly and often results in low cell viability and undesirable transfection efficiency. This paper introduces an efficient, cost-effective, and simple electroporation protocol that demonstrates a high transfection efficiency of ~80–90% and optimal cell viability. This is a simple, reproducible, and efficient genetic modification method that is applicable to the study of the primary cilium-dependent Hedgehog signaling pathway in primary GCP cultures.

INTRODUCTION:

Cerebellar GCPs are widely used to study the machinery of the Hh signaling pathway in neuronal progenitor cell-types owing to their high abundance and high sensitivity to the Hh signaling pathway *in vivo*[1-4]. In GCPs, the primary cilium acts as a pivotal Hh signal transduction hub[5] that

orchestrates the proliferation of the precursor cells[6-8]. *In vitro* visualization of Hh signaling components on the primary cilium is often challenging due to their low endogenous basal levels. Hence, transgene modification of protein expression levels and fluorophore tagging of the gene of interest are useful approaches to study the pathway at molecular resolution. However, genetic manipulation of GCP primary cultures using liposome-based transfection approaches often result in low transfection efficiency, hindering further molecular investigations[9]. Electroporation increases the efficiency but commonly requires exorbitant vendor-specific and cell type-restricted electroporation reagents[10].

This paper introduces a high-efficiency and cost-effective electroporation method to manipulate the Hh signaling pathway components in GCP primary cultures. Using this modified electroporation protocol, a green fluorescent protein (GFP)-tagged Smoothened transgene (pEGFP-Smo) was efficiently delivered to GCPs and achieved high cell survival and transfection rates (80–90%). Furthermore, as evidenced by the immunocytochemical staining, the transfected GCPs showed high sensitivity to Smoothened agonist-induced activation of the Hh signaling pathway by trafficking EGFP-Smo to the primary cilia. This protocol shall be directly applicable and beneficial for experiments that involve *in vitro* genetic modification of cell types that are difficult to transfect, such as human and rodent primary cell cultures, as well as human induced, pluripotent stem cells.

PROTOCOL:

All animal-related procedures were carried out in compliance with animal handling guidelines and the protocol approved by the Department of Health, Hong Kong. Animal experiment licenses following Animal (Control of Experiments) Ordinance (Cap. 340) were obtained from the Department of Health, Hong Kong Government. The animal work was carried out in compliance with the animal safety ethics approved by HKBU Research Office and Laboratory Safety Committee. For optimal GCP yield, use postnatal (P) day 6 or P7 pups for the isolation of GCPs. Refer to the **Table of Materials** for details about all materials used in this protocol.

1. Preexperiment preparation

1.1. Preparation of culture media and buffers

1.1.1. Serum-free medium (SFM)

1.1.1.1. To prepare 50 mL of SFM, add 500 µL of 100x L-glutamine substitute, 500 µL of Penicillin-Streptomycin, 1 mM of sodium pyruvate, and 12.5 µL of 1 M KCl (final 250 µM) to 49 mL of Neurobasal medium.

1.1.1.2. Split the SFM into 2 aliquots of 10 mL and 40 mL in 50 mL conical tubes and store them at 4 °C for up to one month.

1.1.2. Digestion blocking medium: 10% FBS in SFM

1.1.2.1. Add 1.1 mL of heat-inactivated FBS to a 10 mL aliquot of SFM to prepare digestion blocking medium.

1.1.3. GCP culture medium: SFM with B27

1.1.3.1. To prepare GCP culture medium, add 800 µL of serum-free B-27 supplement to a 40 mL aliquot of SFM.

NOTE: B-27-supplemented SFM can be stored at 4 °C for up to one month. However, freshly prepared media produce optimal outcomes.

1.1.4. Dissection buffer: EBSS with glucose + HEPES

1.1.4.1. Add 6 g/L of glucose to calcium- and magnesium-free Earle's Balanced Salt Solution (EBSS) containing 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid).

1.1.4.2. Sterilize the solution by passing through a 0.2 µm syringe filter and store it at 4 °C for long-term storage.

1.1.5. Digestion Buffer

NOTE: Prepare freshly before use.

1.1.5.1. Prepare 2 mL of digestion buffer for the digestion of 2–4 cerebellar tissues and 4 mL for 4–10 tissues. To prepare 4 mL of digestion buffer, dissolve 1.5 mg of L-cysteine (final concentration 200–400 µg/mL) in 4 mL of EBSS. Invert the tube repeatedly until the powder is fully dissolved.

1.1.5.2. Sterilize the solution using a 0.2 µm syringe filter and a 5 mL syringe, and transfer the solution to a sterile 35 mm cell culture dish.

1.1.5.3. Add 4 µL of papain (1:1,000 dilution from a stock concentration of 20 units/mg) and 40 µL of DNase I (1:100 dilution from a 10 mg/mL stock for a final concentration of 0.1 mg/mL).

1.1.5.4. Incubate the solution at 37 °C in a CO_2 incubator for at least 30 min or until use.

NOTE: This step is critical to activate the papain. For optimal outcomes, do not exceed 45 min at 37 °C before use. Ensure the solution turns transparent and there are no white precipitates before use.

1.2. Precoating coverslips

1.2.1. To prepare the coverslips for cell attachment, incubate autoclaved 12 mm glass coverslips with 100 µg/mL of poly-D-lysine (PDL, 1 mg/mL in sterile dH_2O) for at least 1 h at 37 °C. Keep the coverslips in the same PDL solution at 4 °C until use.

1.2.2. On the day of primary cell culture, collect the PDL in a clean conical tube and rinse the coverslips three times with sterile dH_2O thoroughly to remove residual PDL.

NOTE: The PDL can be stored at 4 °C for reuse.

1.2.3. Transfer the PDL-coated glass coverslips onto a 24-well plate by placing one coverslip in each well.

1.2.4. Remove excess water and add 200–300 µL of Matrigel (reconstituted according to the instructions in the product datasheet in serum-free DMEM-F12) to fully cover the coverslips.

1.2.5. Incubate the coverslips in the Matrigel for 1 h at 37 °C in the CO_2 incubator.

NOTE: Remove the Matrigel before cell seeding. This Matrigel can be collected and stored at 4 °C for multiple reuses.

1.3. Preparation of preplating culture dish

1.3.1. Coat a 60 mm cell culture dish with 2 mL of 100 µg/mL PDL (reconstitute the 1 mg/mL PDL stock solution in sterile dH_2O) by incubation at 37 °C for 1 h.

1.3.2. Immediately before cell seeding, remove and collect the used PDL in a clean conical tube and store it at 4 °C for multiple reuses. Rinse and wash the PDL-coated dish three times with sterile dH_2O. Air-dry the culture dish before cell seeding.

1.3.3. Use one 60 mm cell culture dish to seed cells harvested from a maximum of 2 whole-cerebellum tissues.

1.3.4. Prepare additional culture dishes for additional cerebellums.

NOTE: See steps 2.1.18 and 2.1.19 for the use of the abovementioned culture dish.

2. Experimental day 0

2.1. Isolation and culturing of mouse primary GCPs

NOTE: The GCP culture method was modified from a standard protocol, which was briefly described in previous work[6,11-12]. Complete the following dissection steps 2.1.6–2.1.10 as quickly as possible for optimal cell viability.

2.1.1. Prewarm SFM, digestion blocking medium, culture medium, and Opti-MEM at 37 °C during dissection.

2.1.2. On a clean bench, presoak all the dissection apparatus in 70% ethanol for disinfection.

2.1.3. Fill a 60 mm cell culture dish with 2–3 mL of dissection buffer and chill it on ice.

2.1.4. Prepare fresh digestion buffer as described in section 1.1.5 and keep it warm at 37 °C.

2.1.5. Use 70% ethanol to wipe the head of the pup for disinfection.

2.1.6. Decapitate the pup without anesthesia. Use forceps to hold the head and sterilized surgical scissors to cut from the back of the skull to decapitate the pup. Carefully remove the skin and skull to uncover the brain by using forceps.

2.1.7. Use forceps to pinch off the cerebellum and quickly soak it in the pre-chilled dissection buffer prepared in step 2.1.3.

2.1.8. Remove the meninges with the cerebellum submerged in the dissection buffer under a dissecting microscope. Ensure that blood vessel-enriched meninges are seen under the dissecting microscope.

2.1.9. Remove all the meninges using forceps.

NOTE: A cerebellum without meninges should have a whitish appearance.

2.1.10. Remove any visible midbrain tissues on the cerebellum and the choroid plexus at the ventral side of the cerebellum.

2.1.11. Transfer the cerebellum to a 35 mm culture dish prefilled with warm digestion buffer prepared in step 2.1.4. Avoid transferring excess dissection buffer. Cut the cerebellum into fine pieces using microspring scissors as quickly as possible.

2.1.12. Immediately incubate the minced cerebellum at 37 °C for 15 min in a CO_2 incubator. Extend the incubation time to 20 min if more than 4 cerebellums are to be processed.

NOTE: After incubation, the tissue will clump together.

2.1.13. Immediately transfer the digested tissue to the bottom of a new sterile 15 mL centrifuge tube using a P1000 pipette tip prewet with digestion blocking medium (avoid transferring digestion buffer).

2.1.14. Add an appropriate volume of digestion blocking medium (1 mL for 1 cerebellum) to terminate the digestion and pipette up and down gently 30 times using a P1000 micropipette to further dissociate the tissue into a single-cell suspension. Avoid air bubble formation.

2.1.15. Gently pass the cell suspension through a 70 µm cell strainer into a new sterile centrifuge tube to remove cell clumps.

2.1.16. Pass an additional 1 mL of fresh digestion blocking medium through the cell strainer to collect residual cells from the cell strainer.

2.1.17. Centrifuge the filtrate at 200 × *g* for 5 min at room temperature (RT). Remove the supernatant and resuspend the pellet with 1 mL of SFM. Repeat this step twice. Avoid forming air bubbles.

2.1.18. Resuspend the pellet in a final volume of 2 mL of GCP culture medium and transfer the cell suspension to the PDL-coated 60 mm preplating culture dish prepared in step 1.3. Incubate for 15 min at 37 °C in a CO_2 incubator.

NOTE: Do not exceed 20 min.

2.1.19. After incubation, tap the culture dish from the side to dislodge the loosely adherent GCP cells. Collect the adherent GCP cells in culture medium by gentle pipetting using a P1000 pipette. Collect this GCP suspension into a new 15 mL centrifuge tube. Discard the 60 mm dish.

NOTE: Strongly adherent astroglia and fibroblast cells will remain attached on the 60 mm dish bottom and will be separated in this step. Omitting this step will compromise the purity of the GCP culture.

2.1.20. Count the cells and proceed to electroporation immediately.

2.2. Day *in vitro* (DIV) 0: Electroporation for Hh receptor transgene overexpression: pEGFP-Smo

2.2.1. For electroporation using a 2 mm gap cuvette, prepare the following plasmid–cell electroporation mixture for each reaction of electroporation: 1.2×10^6 cells and 10 µg of pEGFP-mSmo (adjust the DNA stock concentration to ~2–5 µg/µL in Tris-EDTA buffer or sterile dH_2O) in 100 µL of Opti-MEM.

NOTE: Reduce the total cell number if the cells are insufficient (though this may reduce the electroporation efficiency). However, do not adjust the amount of plasmid nor the total volume of Opti-MEM used per cuvette. If the total cell number required for the experiment exceeds 1.5×10^6 cells, scale up the electroporation mixture accordingly and perform multiple electroporation reactions separately. The cuvette may be reused up to 5 times.

2.2.2. Prepare the post-electroporation cell seeding plate by adding 0.5 mL of culture medium into each well of the 24-well culture plate containing coated coverslips (from step 1.2) and keep it warm at 37 °C in a CO_2 incubator.

2.2.3. From step 2.1.20, pipette the required number of cells into a sterile 1.5 mL microcentrifuge tube and spin at 200 × *g*for5 min at RT. Discard the supernatant and resuspend the cell pellet in 200 µL of Opti-MEM. Repeat this step twice with Opti-MEM to ensure no residual culture medium is present in the tube

NOTE: For every well/coverslip of a 24-well plate, electroporate and seed 1.2–1.3 × 10^6 cells/well to obtain ~70–75% cell confluency on the next day of culture.

2.2.4. Set the parameters of electroporation as shown in **Table 1**.

2.2.5. Pipette the electroporation reaction gently to mix well and use a long P200 tip to transfer an exact volume of 100 µL of the mixture into the 2 mm gap cuvette. Avoid forming bubbles.

2.2.6. Place the cuvette in the cuvette chamber.

2.2.7. Press the **Ω** button of the electroporator (see the **Table of Materials**) and make a note of the impedance value, which should be ~30–35. To ensure the electric impedance value Ω falls within the range of 30–35, adhere to a precise volume of 100 µL.

2.2.8. Press the **start** button to initiate the pulse.

2.2.9. Record the values of the measured current and joules shown on the reading frame.

2.2.10. Remove the cuvette from the cuvette chamber.

2.2.11. Immediately add 100 µL of prewarmed culture medium into the cuvette and resuspend it by gentle pipetting up and down 2–3 times. Immediately transfer the cell suspension to the 24-well plate prepared in step 2.2.2.

NOTE: To minimize cell death, seed the cells immediately after electroporation.

2.2.12. Incubate the cells at 37 °C in a CO_2 incubator. Leave the cells undisturbed for 3 h to avoid cell detachment.

2.2.13. At 3 h post seeding, gently aspirate and discard half of the supernatant medium to remove floating dead cells and debris and replace with the same amount of prewarmed culture medium.

2.2.14. Incubate the cells at 37 °C in the CO_2 incubator and replenish half of the medium every other day.

2.2.15. Observe the cells under the fluorescence microscope on the next day, i.e., DIV1 (**Figure 1**) for GFP signal to determine the efficiency of Smo overexpression.

2.2.16. For the stimulation of the Hh signaling pathway on DIV 1 after replenishing half of the medium, add 0.2 µM Smoothened agonist (SAG) to the cells while adding an equal volume of DMSO to the control. Incubate for 24 h prior to cell fixation.

NOTE: Keep the volume of DMSO/SAG added as low as possible. Here, 0.5 µL DMSO or 0.5 µL of 0.2 mM SAG was added per 500 µL of medium per well, which is a dilution ratio of 1:1,000.

3. DIV 2: Visualization of primary cilia and investigation of the Hh signaling pathway

3.1. Cell fixation

3.1.1. At 24 h post treatment, remove the culture medium and rinse the cells 2–3 times with phosphate-buffered saline (PBS) using a disposable Pasteur pipette and avoid dislodging the cells.

3.1.2. Fix the cells by adding ~400 µL of 4% paraformaldehyde (prepared in PBS) and incubate at RT for 10 min.

3.1.3. Rinse and wash 2–3 times with PBS using a disposable Pasteur pipette.

3.1.4. Store in PBS at 4 °C for up to 2 months or proceed to immunostaining.

3.2. Immunocytochemical staining of GCPs and primary cilium marker

3.2.1. In a 24-well plate, wash the cells twice in PBS with gentle shaking for 5 min each time.

3.2.2. Add 0.5 mL of 100 mM ammonium chloride to the cell and incubate at RT for 10 min to quench the fixative.

3.2.3. Rinse once and wash 2–3 times with PBS with gentle shaking for 10 min each time.

3.2.4. Gently pipette 30 µL of blocking buffer (BB: 0.1% Triton X-100, 1% BSA, 2% heat-inactivated horse serum in PBS) onto a piece of parafilm to form a droplet without air bubbles.

3.2.5. Gently transfer the coverslip from the well onto the BB droplet with the cells facing downwards. Incubate the cells with BB in a humid chamber for 1 h at RT.

3.2.6. Prepare the primary antibody mix in BB as shown in the **Table of Materials**. To probe the cells with primary antibody, repeat steps 3.2.4 and 3.2.5, replacing BB with the primary antibody mix. Incubate the cells with the primary antibody for 2 h at RT.

3.2.7. Using forceps, transfer the coverslips back to the 24-well plate with the cells facing upwards. Rinse the cells once and wash 3–4 times in PBS with gentle shaking for 10 min each time.

3.2.8. Prepare the secondary antibody mix in BB as shown in the **Table of Materials**. To incubate the cells with the secondary antibody, repeat steps 3.2.4 and 3.2.5, replacing BB with the secondary antibody mix. Incubate in the dark for 1 h at RT.

3.2.9. Repeat step 3.2.7 for post-secondary antibody incubation washing.

3.2.10. Mount the coverslip on a clean microscopic glass slide using mounting medium.

3.2.11. Air-dry the slide in the dark overnight and proceed to confocal imaging[13].

REPRESENTATIVE RESULTS:

Using the Opti-MEM (see the **Table of Materials**) as the universal reagent, this proposed electroporation methodology could achieve consistently high electroporation efficiency at ~80–90% (**Figure 1**). The electroporation efficiency of the Smo-EGFP vector was determined at DIV 2 post electroporation by quantification of the percentage of green fluorescence-positive cells in all paired box protein-6 (Pax6)-expressing GCP cells. The electroporation efficiency of DMSO- and SAG-treated groups appeared comparable (**Figure 1** and **Table 2**).

In addition, immunostaining of the primary cilium marker, Arl13b, demonstrates that the ciliation rate of GCP at DIV 2 of culture was ~18% in both the vehicle- and SAG-treated groups (DMSO: 17.35% ± 0.59%; SAG: 18.24% ± 0.88%). The ciliation rate is illustrated as the percentage of Pax6-expressing GCPs bearing a primary cilium (Arl13b-positive) on the cell surface at DIV 2 post electroporation (**Figure 2** and **Table 3**).

To decipher the primary cilium-dependent Hh signaling pathway, an agonist of Smo, SAG, was used to activate the Hh signaling pathway. Upon Hh pathway activation, the Smo receptor is enriched at the axoneme of the primary cilium[14]. These results show significantly increased Smo-EGFP localization on the primary cilium axoneme of Pax6-expressing GCP cells at 24 h post SAG treatment (**Figure 3,** quantification data modified from previous work[6]), indicating a profound activation of the primary cilium-dependent Hh signaling pathway.

FIGURE AND TABLE LEGENDS:

Figure 1: Electroporation setup and the electroporation efficiency of GCPs. (**A**) Electroporation setup. Right, black arrowhead denotes electroporation cuvette. (**B, C**) Representative images depict the electroporation efficiency of the Smo-EGFP vector determined by quantification of the percentage of GFP-positive cells in all Pax6-expressing GCP cells (**Table 2**). Representative images depict the green fluorescent signals on Pax6-expressing (violet) GCP cells on DIV 2 post electroporation after 24 h treatment with (**B**) DMSO and (**C**) SAG. Nuclei were labeled with DAPI

(blue). Scale bars = 20 µm. Abbreviations: GCPs = granule cell precursors; GFP = green fluorescent protein; Pax6 = paired box protein-6; DIV = day *in vitro*; DMSO = dimethyl sulfoxide; SAG = Smoothened agonist; DAPI = 4',6-diamidino-2-phenylindole.

Figure 2: Percentage of ciliation on DIV 2 of GCP primary culture. (**A, B**) Representative images depict the percentage of ciliation on DIV 2 of GCP primary culture. Representative images depict the primary cilia (green) on Pax6-expressing (red) GCP cells on DIV 2 post electroporation after 24 h treatment with (**A**) DMSO and (**B**) SAG. Nuclei were labeled with DAPI (blue). The primary cilium (green) is denoted by white arrowheads. Scale bars = 20 µm. (**C**) Graph illustrates quantification data of 4 independent experiments. Statistical analysis, Unpaired Student's *t*-test. Error bars depict ±SEM. Abbreviations: GCP = granule cell precursor; Pax6 = paired box protein-6; DIV = day *in vitro*; DMSO = dimethyl sulfoxide; SAG = Smoothened agonist; DAPI = 4',6-diamidino-2-phenylindole; n.s. = Not significant; SEM = standard error of the mean; Arl13b = ADP ribosylation factor-like protein 13B.

Figure 3: Increased Smo localization on the primary cilium of Pax6-expressing GCP cells upon SAG treatment. (**A**) Representative images depict the Smo-EGFP localization (green) on the primary cilium (red, white square box) on Pax6-expressing (violet) GCP cells at DIV 2 post electroporation after 24 h treatment with DMSO and SAG. Nuclei were labeled with DAPI (blue). Scale bars = 5 µm. (**B**) Graph illustrates quantification data of 4 independent experiments. Statistical analysis, unpaired Student's *t*-test. *** $P \leq 0.001$. Error bars depict ± SEM. Total n for DMSO group = 97, total n for SAG group = 130. **Figure 3B** was modified from [6]. Abbreviations: GCP = granule cell precursor; Smo = Smoothened; Pax6 = paired box protein-6; DIV = day *in vitro*; DMSO = dimethyl sulfoxide; SAG = Smoothened agonist; DAPI = 4',6-diamidino-2-phenylindole; SEM = standard error of the mean; Arl13b = ADP ribosylation factor-like protein 13B.

Table 1: The electroporation parameters of mouse primary GCPs and primary neurons using Super Electroporator NEPA21 TYPE II. Abbreviation: GCP = granule cell precursor.

Table 2: Electroporation efficiency of Smo-EGFP vector determined by quantification of the percentage of GFP-positive cells in all Pax6-expressing GCP cells. Data of four independent experiments (Exp.) are shown. (Total n = 2980). Abbreviations: Smo = Smoothened; GFP = green fluorescent protein; GCP = granule cell precursor; Pax6 = paired box protein-6.

Table 3: The percentage of ciliation on DIV 2 of GCP primary culture. Data of four independent experiments (Exp.) are shown. (Total n for DMSO group = 1169, Total n for SAG group = 816). Abbreviations: GCP = granule cell precursor; DIV = day *in vitro*; DMSO = dimethyl sulfoxide; SAG = Smoothened agonist.

DISCUSSION:

Transfection of transgenes in primary GCP culture by electroporation method is typically associated with low cell viability and poor transfection efficiency[9-10]. This paper introduces a cost-effective and reproducible electroporation protocol that has demonstrated high efficiency and

viability. In addition, we also demonstrate a straightforward method of studying the primary cilium-dependent Hh signaling pathway in primary GCP cells.

Other common electroporation methods often require costly cell-type-specific electroporation reagents that must be purchased from specific manufacturers. The method described here is deemed favorable as it uses a common and economical electroporation reagent for different cell types. Moreover, these data showed that the electroporation efficiency reached ~80–90%, which is highly efficient compared to other electroporation and transfection methods[9-10].

To maintain higher cell viability, there are a few critical steps that one should take into consideration. The cerebellum dissection and dissociation procedures should be completed within the shortest possible time window of 1–2 h. Another critical factor is to avoid bubble formation falls in the plasmid–cell electroporation mixture before pulses during electroporation. After pulses, prewarmed culture medium should be added immediately into the cuvettes and the cells seeded as quickly as possible. The cells must be undisturbed in the first 3 h post cell seeding. The aforementioned precautions will enhance cell viability up to approximately 70–80% on the second day of culture.

One notable limitation of studying the primary cilium in the primary culture platform is that the rate of ciliation in cultured cells is generally lower than that observed *in vivo*. Previous data [6] showed that the *in vivo* rate of ciliation on GCP at both E15.5 and P15 was approximately 60–80%. In contrast, the *in vitro* rate of ciliation in primary GCP culture was ~20%[6]. Nonetheless, this is a general phenomenon that is discernible across most (if not all) cell types when comparing the rate of ciliation between *in vitro* and *in vivo* studies.

Notably, this method is also applicable to other primary cultures such as neural progenitor cells and cortical and hippocampal neuron culture, which is achievable by modifying the electroporation parameter, i.e., poring pulse voltage, length, and number of pulses. To extend the application of this protocol to a broader field of study, the recommended electroporation parameters for primary neurons are provided in **Table 1**. In addition, the universal electroporation reagent, i.e., Opti-MEM used in this protocol also helps avoid additional tedious optimization effort compared to other electroporation protocols that require optimization with respect to reagent compatibility. This optimized, cost-effective electroporation protocol for the investigation of the primary cilium and Hh signaling in primary GCP cultures could be used as the reference procedure for other primary cilium-related studies using primary cultures.

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DISCLOSURES:
The authors have no conflicts of interest to declare.

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Figure

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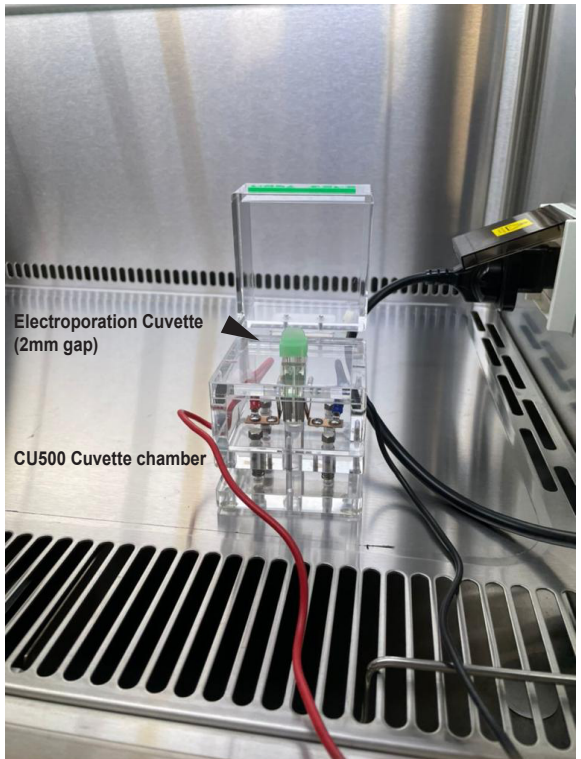

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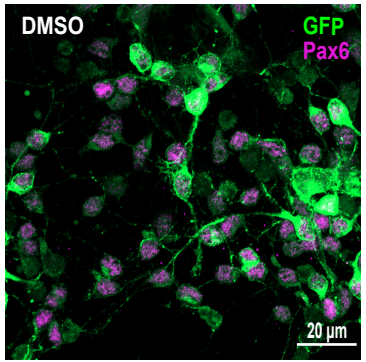

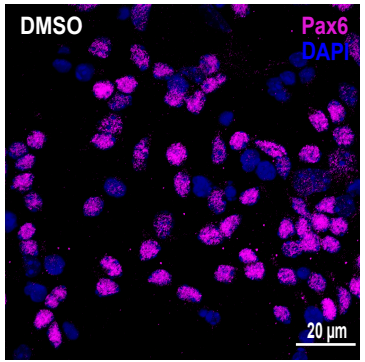

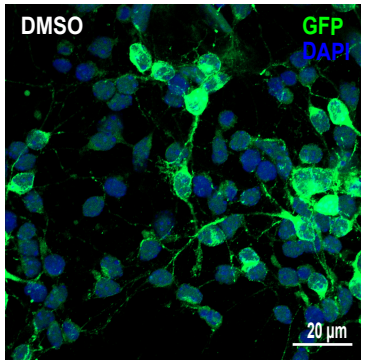

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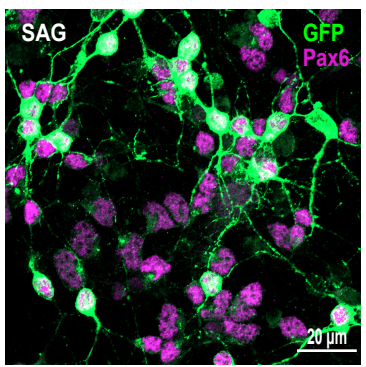

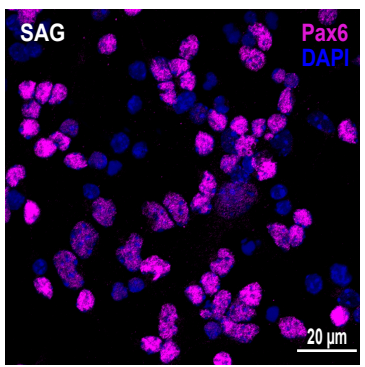

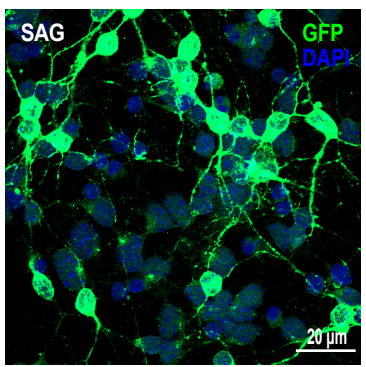

Figure

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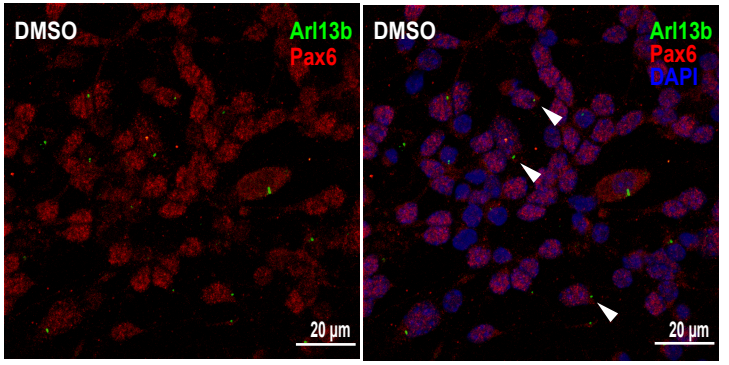
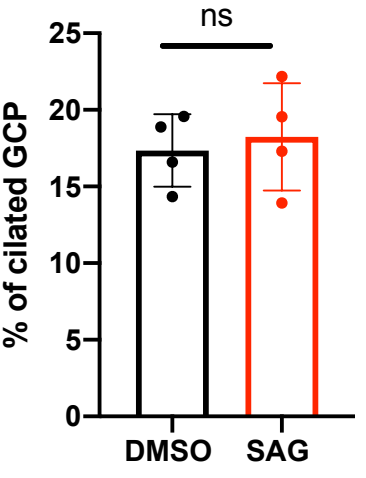
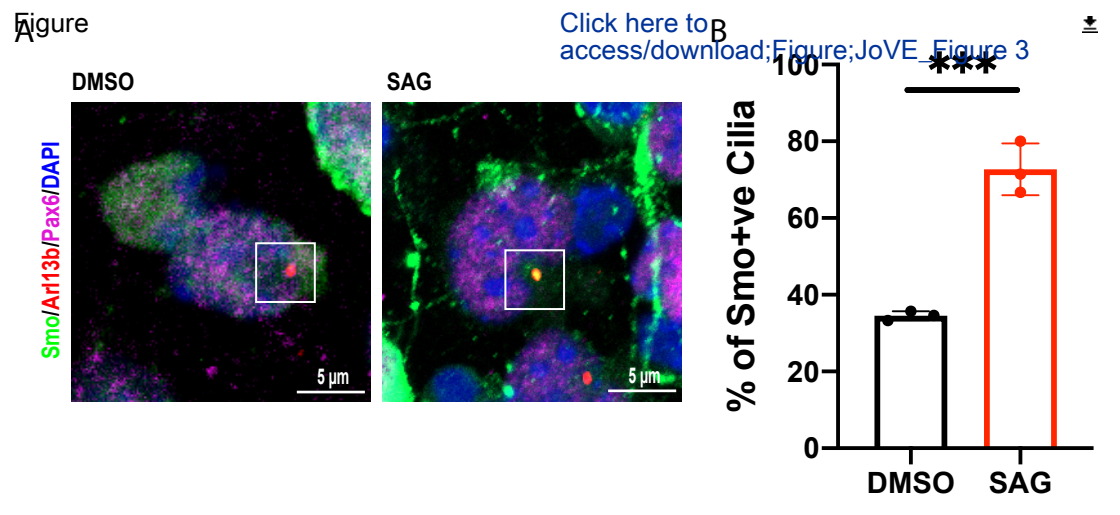




Figure

Click here to access/download;Figure;JoVE_Figure 3



	Poring Pulse Setting		Transfer Pulse Setting	
	Mouse Primary GCPs	**Primary neurons**	**Mouse Primary GCPs**	**Primary neurons**
Voltage	275 V	275 V	20 V	20 V
Length	1 ms	0.5 ms	50 ms	50 ms
Interval	50 ms	50 ms	50 ms	50 ms
No.	2	2	5	5
D rate	10%	10%	40%	40%
Polarity	+	+	±	±

Table 2

Click here to access/download;Table;JoVE_Table 2_Electroporation Efficiency_final.xlsx

Electroporation efficiency	Exp. 1 (n = 486)	Exp. 2 (n = 1314)	Exp. 3 (n = 704)	Exp. 4 (n = 476)	Average
DMSO-treated group	90.57% ± 10.12%	96.62% ± 3.09%	98.89% ± 0.97%	90.72% ± 11.31%	94.02% ± 1.36%
SAG-treated group	91.8% ± 8.69%	79.97% ± 2.77%	89.35% ± 5.67%	88.59% ± 13.54%	87.42% ± 1.71%
Average Electroporation efficiency	91.31% ± 7.99%	88.27% ± 10.81%	94.12% ± 6.36%	89.65% ± 11.21%	90.84% ± 0.84%

Table 3

	Exp. 1	Exp. 2	Exp. 3	Exp. 4	average
Ciliation rate - DMSO	18.88% ± 3.61%	19.58% ± 7.42%	16.60% ± 1.48%	14.35% ± 7.99%	17.35% ± 0.59%
Ciliation rate - SAG	13.93% ± 3.39%	17.30% ± 2.15%	22.19% ± 10.35%	19.56% ± 1.15%	18.24% ± 0.88%

All the editor's comments including figure legends, proper formatting, additional info for discussion and introduction sections and so on have been addressed in the text with tracking records.

Responses to reviewers' comments:

Reviewer #1:

The manuscript entitled, "Efficient and cost-effective electroporation method to study primary cilium-dependent Hedgehog signaling pathway in the primary cerebellar granule precursor culture" by Lo et al succinctly describe the method of in vitro electroporation of plasmid DNA into cultured granule cell progenitors from the cerebellum. The protocol is very nice and will be a good addition to others describing in vitro electroporation. In general, I am very enthusiastic about that protocol, however I have comments that should be addressed.

Major

1. Is the electroporation efficiency in Figure 1 80-90%? Have you quantified this? A graph representing this number with Std. Dev. should be presented.

   Yes. The quantification of the electroporation efficiency with standard deviation of multiple independent experiments was listed in **Table.3**.

2. Is this a percentage of all surviving cells or is there cell death from the point of electroporation? Estimates should be included based on the number of cells placed into the cuvette.

   Yes. There was cell death at the point of electroporation. We observed approximately 20-30% of cell death on the next day post-electroporation. The critical points to take note to enhance cell viability have been described at **steps 2.11-2.14, part B** and discussion.

3. Does electroporation alter the rate of ciliation?
   From our previous analyses on the rate of ciliation on the non-electroporated GCP cultures showed about 20% of ciliated cells (Fig. 6E, Hor et al. 2021 *Journal of Neuroscience* 41(32): 6850–63). In the present work, the average percentage of ciliation of GCP cells after electroporation was 17.35% ± 0.59%. These observations suggest that electroporation is unlikely to have a huge impact on the rate of ciliation.

4. How does the rate of ciliation in your experiment compare to in vivo?

   The rate of ciliation in culture is generally lower than that observed *in vivo*. This is a general phenomenon that is discernible across most (if not all) cell types when comparing the rate of ciliation *in vitro* and *in vivo*. For stable cell line culture, the most widely reported approach to enhance the rate of ciliation *in vitro* is performing a 24-

48 hours serum starvation. Our previous data (Fig. 6D-E, Hor et al. 2021 *Journal of Neuroscience* 41(32): 6850–63) showed that the in vivo rate of ciliation on GCP at both E15.5 and P15 was approximately 60-80%. Whereas, the *in vitro* rate of ciliation on primary GCP culture was around 20%.

We have now mentioned the issue concerned in the discussion section.

5. Are there plasmids that can be used to label only the primary cilia?

   Yes. Arl13b.

6. A general statement is that you indicate that your protocol is cost effective, but no comparisons are given. How expensive is the protocol in comparison to others? I would imagine the electroporator and cell culture reagents are quite expensive. Statements regarding cost and protocols need to be substantiated or reworded.

   In order to avoid potential improper infringement and conflict of interest, we are not able to explicitly mention and/or compare the cost between different brands at this platform. However, from our local list price obtained from local distributors, the cost of the electroporator used in this protocol is comparable to other electroporators that are commonly used for neuron and primary cell culture work. In addition, the electroporation reagent used in this protocol, Opti-MEM is commonly used and universal. Besides, the cost of Opti-MEM is substantially more economical than other cell type specific electroporation reagents used in other brands.

Minor

"The investigation of primary cilium dependent Hh signaling machinery requires in vitro over-expression of the transgene of the pathway components to visualize the localization of the Hh pathway receptors and transcription factors on the primary cilium."

1. Please change the word over-expression to manipulation. Also, there are other ways to manipulate/label the cilia, for example using viruses and transgenic mice as well as using CRE, and CRISPR/Cas technologies that do not necessarily use over-expression but do allow for endogenous labeling. Thus, the word requirement is perhaps not appropriate. Moreover, one can perform immunostaining to visualize the primary cilia.
   We changed the word to manipulation.

2. The following sentence requires a reference, "However, genetic manipulation of primary GCP culture using
   45 the liposome-based transfection approach is often challenging due to the low transfection
   46 efficiency that hinders further molecular investigations."

   References are now added accordingly..

3. It is unclear whether all appropriate animal ethics boards approved the protocol. The authors state "All animal related procedures were carried out in compliance to animal handling guidelines and 105 protocol approved by the Department of Health, Hong Kong.". Please check to determine whether additional approval statements are required (In the US there is also an IACUC approval in addition to external granting agencies and laws).

   We are grateful to the reviewer for mentioning this important point. Indeed, we ought to clarify the protocol approvals appropriately.
   All experiments fulfilled all relevant legislation and Codes of Practice in Hong Kong. Animal experiment licences following Animal (Control of Experiments) Ordinance (Cap. 340) were obtained from the Department of Health, Hong Kong Government. The animal works were carried out also in compliance with the animal safety ethics approved by HKBU Research Office and Laboratory Safety Committee.

4. 134 1.12 After incubation, the tissue will appear slimy and clump together. Is there a more appropriate word than slimy?

   Many thanks for the comment and suggestion. We changed to 'sticky'.

Reviewer #2:

1. Most of the electroporation methods used for transfection of neurons require expensive cell type-specific electroporation reagents. In this ms, the authors introduce an efficient, cost-effective, and simple electroporation protocol that gives high transfection efficiency at ~80-90%, and optimal cell viability in GCP. This is very impressive and the method should be published to save the cost for labs in the study of neurons. The authors mentioned that "this method is also applicable to other primary cultures such as neural progenitor cells, cortical and hippocampal neuron culture, which is achievable by modifying the electroporation parameter i.e. poring pulse voltage, length, and number of pulses".

   I would recommend that the authors provide the details for them in a table as that in Table 1 for different types of neurons listed above. In this way, more laboratories will benefit from this study.

   Many thanks for the constructive comments. The electroporation parameters for primary neurons are now listed in **Table 5.**

Reviewer #3:

Manuscript Summary:
This protocol described an electroporation method to efficiently transfect granule neuron precursors from the developing cerebellum. The resulting high transfection efficiency and high viability makes this protocol very interesting to the scientific community. The protocol included step-by-step description with enough details, and easy to follow.

Major Concerns:

1. The title does not exactly match the content of the protocol. The protocol has little to do with the Hh pathway and the primary cilium. Under such a high expression level of Smo-GFP as described in figure 1, most of Smo will be located in the primary cilium without SAG treatment. The suggestion is to change the title to emphasize the interesting high efficiency and high viability, and understate the primary cilium and Hedgehog signaling.

We truly appreciate the discussion concerning the appropriateness of the title. Besides sharing a cost-effective and efficient electroporation procedure for primary GCPs that is beneficial to a broad scientific community, we hope the readers could appreciate that the protocol is also comprehensive and useful for the researchers studying Hh signaling and primary cilium. In most (if not all) in vitro experimental platforms employed to study the Hh signaling, the basal level of Hh pathway components are hardly visible or detectable on the primary cilium. This phenomenon is similarly observed in primary GCP culture, our data from Fig 1 shows that upon over-expression, the percentage of Smo+ve cilia detectable is at 30-40%. Note that this 30-40% of Smo-expressing cilia was counted from 15-20% of ciliated cells. Without overexpression, one may possibly find (if any) Smo+ve primary cilia at 10% or lower, from the total number of GCP, which is extremely difficult to achieve a decent and reasonable analysis. Hence, we believe that our protocol not only offers a cost-effective electroporation procedure, but also an effective method of studying primary cilium and ciliary signaling pathways in GCPs. Nevertheless, we have now modified the title to - Efficient and cost-effective electroporation method to study primary cilium-dependent Hedgehog signaling in primary cerebellar granule precursor culture.

Minor Concerns:

1. The author should discuss why a pre-plating step is needed in this protocol. Can this step be skipped? How will it impact the final result if this step is skipped?

   The purpose of the pre-plating step was mentioned at step 1.18. which serves to separate/remove unwanted astrocytes and fibroblasts. We have now added additional comments on the unfavourable outcome if one omits this step.

2. The papain concentration (stock and final) needs to be described.

   We thank the reviewer for pointing out the missing information. The concentration of papain (20 units/mg) is now added to the text.

香港浸會大學
HONG KONG BAPTIST UNIVERSITY

Catherine Hong Huan HOR <catherinehor@hkbu.edu.hk>

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2 messages

Catherine Hong Huan HOR <catherinehor@hkbu.edu.hk> Tue, Nov 2, 2021 at 12:10 AM
To: jnejpsite@gmail.com, jneurosci@msubmit.net, jn@sfn.org

Dear editor,

Good day.

I am writing to seek permission to reuse (with citation) a part of our raw data recently published in The Journal of Neuroscience as the supporting data for publication in our prospective protocol manuscript to be published in the protocol journal, JoVE.

In our previous published work titled, 'Multifaceted functions of Rab23 on primary cilium- and Hedgehog signaling-mediated cerebellar granule cell proliferation' (DOI: https://doi.org/10.1523/JNEUROSCI.3005-20.2021), we studied primary cilium-dependent Hh signaling pathway on *in vivo* and *in vitro* GCP culture. In this work, part of the results were produced from a newly optimized protocol that we established in our own lab. In particular, we developed a cost-effective in vitro electroporation method for genetic manipulation in primary GCP culture.

We believe that sharing this optimized cost-effective protocol would be beneficial to the neuroscience community. Given that our newly established protocol was built in parallel with the work that we published in The Journal of Neuroscience, we would like to cite and reuse our published data as the evidence to support the success of this new protocol.

For this reason, we would like to seek your official permission to reuse one set of raw data (i e. Figure 7D-E, partial data with modification, DOI: https://doi.org/10.1523/JNEUROSCI.3005-20.2021) originating from the JN paper for our prospective publication in the protocol journal the JoVE.

Our manuscript for JoVE publication is tentatively titled: "Efficient and cost-effective electroporation method to study primary cilium-dependent Hh signaling pathway in the primary granule precursor culture,"

Appreciate your time and kind consideration on this copyright permission.

We look forward to receiving your favorable response soon.

best regards,

Catherine Hor (Corresponding author)

On Wed, Jun 23, 2021 at 12:07 PM JNeurosci <jneurosci@msubmit.net> wrote:

> 23rd Jun 2021
>
> Dear Dr. HOR:
>
> I am pleased to inform you that your revised paper, "Multifaceted functions of Rab23 on primary cilium- and Hedgehog signaling-mediated cerebellar granule cell proliferation," has been accepted for publication in The Journal of Neuroscience.
>
> Your manuscript will now pass through a series of preproduction checks. Once your manuscript is completely ready and the publication fee has been paid, you will receive email notification that it has been forwarded to production.
>
> You will receive a link to the invoice and payment portal within 48 hours. Please note that proofs will not be sent

> to you until 2-3 weeks after and the article will not publish until the publication fee has been received in full.
>
> Publication fees are reduced if the corresponding author is a member of the Society for Neuroscience. The publication fee is $1,535 for members and $2,185 for non-members. If you would like to join SfN, please visit our Member Center at https://www.sfn.org/membership. A revised invoice will be sent 24-48 hours after your membership is updated. If you do not receive an updated invoice within that timeframe, please contact us at jn@sfn.org.
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> On behalf of the Editorial Board, thank you for submitting this paper to The Journal of Neuroscience.
>
> Yours sincerely,
>
> Dan Sanes
> Senior Editor
> Journal of Neuroscience

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jn permissions <jnpermissions@sfn.org> Fri, Nov 5, 2021 at 2:35 AM
To: Catherine Hong Huan HOR <catherinehor@hkbu.edu.hk>

Hello Dr. Hor,

Thank you for contacting us. Since this is your previous work, no special permissions are needed.

Regards,

Vicente Carmona

Editorial Associate I

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