Protocol
HIPRO: A High-Efficiency, Hypoxia-Induced Protocol for Generation of Photoreceptors in Retinal Organoids from Mouse Pluripotent Stem Cells

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HIGHLIGHTS
Efficient generation of retinal organoids from mouse stem cells using hypoxia
Improved photoreceptor biogenesis in organoid cultures
Development of Nrl-GFP tagged rod photoreceptors evaluated by gene profiling
Protocol
HIPRO: A High-Efficiency, Hypoxia-Induced Protocol for Generation of Photoreceptors in Retinal Organoids from Mouse Pluripotent Stem Cells

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SUMMARY
Mouse pluripotent stem cells can be efficiently differentiated into retinal organoids with polarized, laminated neural retina harboring all retinal cell types by the Hypoxia-Induced Generation of Photoreceptor in Retinal Organoids (HIPRO) protocol. In our recent publication, we modified the HIPRO protocol on the basis of comparative transcriptome analyses to facilitate photoreceptor biogenesis and maturation. Here, we provide a detailed protocol for efficient generation of retinal organoids from mouse pluripotent stem cells. For complete details on the use and execution of this protocol, please refer to (Chen et al., 2016, DiStefano et al., 2018, Brooks et al., 2019).

BEFORE YOU BEGIN
Preparation of Reagents, Culture Media and Cultureware

Δ CRITICAL: Except weighing and pH adjustment, perform all other procedures in a Biological safety cabinet Class II to ensure sterility.

Reconstitution of Reagents

Θ TIMING: 2.5–3 h

0.1 M taurine (100x)

1. Weigh 0.5 g taurine powder and add 40 ml ultrapure water.
2. Gently mix the taurine solution on a shaker for ~1 hour to completely dissolve the powder.
3. Filter the taurine solution with a 0.22 μm syringe filter.
4. Store the filtered 0.1 M taurine solution at 4°C for up to 1 year.

Δ CRITICAL: Use only sterile ultrapure water for organoid cultures.

10 μg/mL insulin-like growth factor 1 (IGF1, 500x)
1. Briefly vortex and spin down 100 μg IGF1 powder.
2. Add 1 mL ultrapure water into the tube to prepare 100 μg/mL solution. Mix well and spin briefly.
3. Aliquot 0.9 mL DMEM/F12 into ten 1.5 ml microfuge tube.
4. Add 0.1 mL 100 μg/mL IGF1 solution to each tube. Mix well by inverting the tube multiple times.
5. Spin down the tubes and store them at –20°C.

Note: 10 μg/mL IGF1 solution is stable at –20°C for half a year. Once thawed, store at 4°C where it is stable for 1 week.

10 mM 9-cis retinal

1. Gently tap 25 mg 9-cis retinal powder to remove the powder that is stuck to the cap.
2. Add 1.25 mL DMSO to the powder to prepare 70 mM stock. Mix well by pipetting up and down several times.
3. Aliquot the 70 mM stock in 1.5 ml amber tubes, 100 μl/tube.
4. Prepare 10 mM working solution by mixing 10 μl stock with 60 μl DMSO.
5. Mix well by pipetting up and down several times.

△ CRITICAL: Reconstitute 9-cis retinal under dim light environment

△ CRITICAL: Store 70 mM stock solution at –80°C for up to one year. The 10 mM working solution is stable at –20°C for half a year.

200 μg/mL fibroblast growth factor 1 (FGF1, 1000x)

1. Dissolve 13.8 g of NaH₂PO₄·H₂O in H₂O to make a final volume of 100 mL. This yields 1 M NaH₂PO₄ solution.
2. Dissolve 14.2 g of Na₂HPO₄ in H₂O to make a final volume of 100 mL. This yields 1 M Na₂HPO₄ solution.
3. Mix 193.5 μL 1M NaH₂PO₄ and 56.6 μL 1M Na₂HPO₄. Adjust pH to 7.4 by NaOH solution or phosphoric acid. Add sufficient H₂O to make a final volume of 50 mL. This yields 5 mM sodium phosphate buffer, pH 7.4.
4. Filter the 5 mM sodium phosphate buffer using a 0.22 μm syringe filter. Store at 4°C.
5. Gently tap 100 μg FGF1 powder to remove the powder that is stuck to the cap.
6. Add 0.5 mL 5 mM sodium phosphate buffer, pH 7.4, to the powder to prepare 200 μg/mL stock solution. Mix well by pipetting up and down several times.
7. Aliquot the stock in 1.5 ml microfuge tubes, 25 μl/tube.

△ CRITICAL: Store at 4°C after thawing for up to 1 week. Avoid repetitive freeze and thaw.

8.5 μg/ml Docosahexaenoic acid (DHA, 2.6 mM, 100x)

1. Weigh 0.4383 g NaCl powder and add sufficient H₂O to make a final volume of 50 mL. This yields 150 mM NaCl solution.
2. Weigh 0.375 g fatty acid-free bovine serum albumin (BSA) power and add sufficient H₂O to make a final volume of 50 mL. This yields 7.5 mg/mL BSA solution.
3. Filter the 150 mM NaCl solution and 7.5 mg/mL BSA solution using a 0.22 μm syringe filter. Store at 4°C.
4. Gently tap 100 mg DHA powder to remove the powder that is stuck to the cap.
5. Add 1 mL 95% Ethanol to prepare a 100 mg/mL DHA stock solution.
6. Add 39 mL filtered 150 mM NaCl solution to make a 2.5 mg/mL stock.

7. Mix 6.8 µL 2.5 mg/mL DHA stock with 1.993 mL 7.5 mg/mL BSA solution in a 15-mL centrifuge tube.

**Note:** The volume of DHA stock and BSA solution can be adjusted according to needs.

8. Wrap the tube with parafilm and aluminum foil. Incubate in 37°C water bath for 30 min. Invert the tube every 5–10 min.

9. Transfer 1.5 mL conjugated BSA-DHA solution to amber 1.5 mL tube to drive away air and avoid oxidation.

10. Add 3 uL 55 mM β-mercaptoethanol to each tube. Mix well by inverting the tube several times.

11. Wrap the tube with parafilm and store at −20°C.

**Aliquot Matrigel**

© TIMING: 30 min

**Note:** Matrigel concentration is critical for proper differentiation. Typically, 2% (vol/vol) Matrigel with a concentration >9.5 mg/mL is added to each well of 96-well plates. Matrigel aliquots of 240 µL are made in 1.5 mL microfuge tubes. However, there is lot-to-lot variation in Matrigel concentration. The volume of Matrigel in each aliquot is adjusted according to the lot concentration, which is available on Corning Life Sciences website under the “Download Certificates” tab.

1. One day before aliquoting, thaw a bottle of Matrigel in a covered polystyrene ice bucket full of ice. Store the box at 4°C. Chill the P1000 tips, 1.5 mL microfuge tubes, and containers for storing the Matrigel aliquots at −20°C.

2. Mix Matrigel by inverting the bottle several times.

3. Using chilled tips and microfuge tubes, make aliquots according to the calculated volume as quickly as possible. Immediately put the aliquots into ice.

4. Store the aliquots at −80°C. The aliquots can be kept at −80°C till the expiration date.

**Coating Poly 2-hydroxyethyl methacrylate (polyHEMA) Cultureware**

© TIMING: 6–16 h

1. Dissolve 2 g polyHEMA powder in 100 mL 95% ethanol. Incubate at 65°C with frequent mixing until the powder completely dissolves. The resulting 20 mg/mL solution can be stored at 22–25°C indefinitely.

2. Rinse non-tissue culture (TC)-treated cultureware with sufficient polyHEMA solution to cover the bottom and edges. Unused solution can be returned back to the stock.

**Note:** We commonly use 6-well plates, 60 mm dishes or 100 mm dishes. Cultureware of other size can also be used depending on the scale of cultures.

3. Dry the plates with cap open for 12–16 hours (minimum 2–3 hours) at 22–25°C under UV light to ensure sterility.

4. Wrap the cultureware with parafilm to maintain sterility. Coated cultureware can be stored at 22–25°C for up to 1 year.
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| RHO                 | A gift from Dr. Robert Molday, University of British Columbia, Canada | n/a |
| OPN1SW              | Santa Cruz | sc-14363 |
| CHX10               | Abcam | ab16142 |
| PKCz                | Sigma | P4334 |
| RCVRN               | Chemicon | AB5585 |
| GS                  | Millipore | MAB302 |
| BRN3A               | Millipore | MAB1585 |
| CALB                | Calbiochem | PC253L |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix | Corning | 354230 |
| ESGRO® Leukemia Inhibitory Factor (LIF) | Millipore | ESG1107 |
| 9-cis retinal       | Millipore | R5754 |
| Sodium pyruvate solution | Millipore | S8636-100ML |
| β-Mercaptoethanol   | ThermoFisher | 21985023 |
| MEM Non-essential Amino Acid Solution (100X) | Millipore | M7145 |
| B-27® Supplement (SOX), minus vitamin A | ThermoFisher | 12587010 |
| DMEM                | ThermoFisher | 11995073 |
| DMEM/ F12 with GlutaMax | ThermoFisher | 10565042 |
| Fetal Bovine Serum  | ThermoFisher | 16000069 |
| Embryonic stem cell-qualified Fetal Bovine Serum | ThermoFisher | 16141079 |
| G-MEM               | ThermoFisher | 11710035 |
| GlutaMAX™ Supplement | ThermoFisher | 35050061 |
| IGF1 Recombinant Human Protein | ThermoFisher | PHG0071 |
| Knockout™ DMEM      | ThermoFisher | 10829018 |
| N-2 Supplement (100X) | ThermoFisher | 17502048 |
| Penicillin-Streptomycin (10,000 U/mL) | ThermoFisher | 15140122 |
| TripLE Express      | ThermoFisher | 12604021 |
| 0.05% Trypsin-EDTA  | ThermoFisher | 25300054 |
| Bovine serum albumin | Millipore | A7030 |
| Fibroblast growth factor 1 | PeproTech | 100-17A |
| Docosahexaenoic acid | Millipore | D2534 |
| UltraPure™ DNase/RNase-Free Distilled Water | ThermoFisher | 10977015 |
| Poly(2-hydroxyethyl methacrylate) | Millipore | P3932 |
| Experimental Models: Cell Lines | | |
| Nrl-GFP wildtype ESCs and iPSCs | NNRL/NEI/NIH | n/a |
| Other               |        |            |
| 25cm² Cell Culture Flask | Corning | 430639 |

(Continued on next page)
Reconstitution of Culture Media

Note: Unless specified, filter all reagents through low protein binding polystyrene membrane with 0.22 μm pore size and keep in fridge. Use these reagents within one month unless indicated otherwise.

Note: Refer to Key Resources Table for a complete list of materials and equipment. If alternative suppliers are preferred, we highly recommend comparing the quality of differentiation with the ones using the designated suppliers at the first several batches of experiments.

△ CRITICAL: Thaw all reagents at 4°C or 22–25°C. Avoid multiple freeze/thaw.

△ CRITICAL: Mix reagents according to the order in which they are listed.

Embryonic Stem Cell (ES) Medium

| Ingredient                               | Volume      | Final concentration |
|------------------------------------------|-------------|---------------------|
| Knockout™ DMEM                           | 500 mL      | n/a                 |
| Non-essential amino acid (100x)         | 6 mL        | 1%                  |
| Penicillin-streptomycin (100x)          | 6 mL        | 1%                  |
| GlutaMAX (100x)                          | 6 mL        | 1%                  |
| Embryonic stem cell FBS                  | 90 mL       | 15%                 |

Maintenance Medium

| Ingredient                          | Volume     | Final concentration |
|-------------------------------------|------------|---------------------|
| ES medium                           | 50 mL      | n/a                 |
| Leukemia inhibitory factor (LIF)    | 10 μL      | 2000 U/mL           |

Note: DO NOT filter maintenance medium. After addition of LIF, the medium can be kept in the fridge for ONE WEEK only.

Freezing Medium

| Ingredient                        | Volume | Final concentration |
|-----------------------------------|--------|---------------------|
| FBS                               | 9 mL   | 90%                 |
| Dimethyl sulfoxide (DMSO)         | 1 mL   | 10%                 |

Note: Freshly prepared before use. Keep at 4°C for at least 5 min after reconstitution as the mixing generates heat.
STEP-BY-STEP METHOD DETAILS
We typically use mouse pluripotent stem cells from passage (P)11 to P25 for differentiation. At least three passages are needed after cell revival for efficient differentiation. For routine maintenance of pluripotent stem cells lines, please follow Steps 1–9 of D0 experiments. Mouse pluripotent stem cells are cultured in maintenance medium with a density of 60%–80% in T25 flask intended for Day 0. Reduce manipulation time as much as possible to alleviate cellular stress and to avoid degradation of reconstituted media.

Day 0: Cell Dissociation and Seedling
© TIMING: 1.5 h

1. Add β-mercaptoethanol (2-ME) to ES medium, maintenance medium, and retinal differentiation medium to make a final concentration of 55 μM.
2. Aspirate ES medium from T25 flasks, wash once with PBS.
3. Rinse flasks with 1 mL prewarmed TrypLE and incubate with another 1 mL TrypLE for 3 min at 37°C.

△ CRITICAL: Check the flasks at 2 min 40 s. If most cells detach from the flasks after gentle tapping, proceed to the next step immediately. DO NOT exceed 3 min. Prolonged incubation with TrypLE is detrimental to the cells.
Alternatives: If some cell lines appear to be more difficult to dissociate, 0.05% Trypsin can be used as a substitute.

4. Add 6 mL ES medium into each flask, gently pipet up and down for 15 times, and transfer the cells to a 15-mL centrifuge tube.

△ CRITICAL: Avoid bubbles during pipetting.

Note: Mouse pluripotent stem cells may be difficult to dissociate in the first or two splits after revival. Use 70μm Cell Strainer to remove cell clumps after pipetting. DO NOT extend the incubation time with dissociating reagents.

5. Centrifuge at 180 g for 5 min at 22–25°C. If feeder-free culture system is used, aspirate the supernatant and proceed to Step 9.

Note: We typically use a feeder system for mouse pluripotent stem cells for maintaining cell quality and pluripotency. Multiple passages are required to adapt mouse pluripotent stem cells from feeder to feeder-free system, especially for induced pluripotent stem cells, and the differentiation capacity may be altered after the adaptation. We note that commercially available feeder cells are expensive, the generation of feeder cells from fibroblasts is labor-intensive, and the use of feeder cells brings further variations to stem cell cultures. The reader can consider which system to use based on budget, cell quality, stability and pluripotency, as well as the workload.

6. If feeder-culture system is used, resuspend the pellet in 4 mL of maintenance medium.
7. Transfer the cells to a T25 flask and incubate at 37°C for 20 min to adhere feeder cells to the flask.
8. Collect the medium containing the floating cells from the flask into a 15-mL centrifuge tube.
9. Add 0.25 x 10^6 (for 3-day culture) or 0.5 x 10^6 cells (for 2-day culture) in 8 mL of maintenance media to a new T25 flask to maintain the cell line.

Note: Use T25 flask with feeder cells for feeder-system cultures. For feeder-free system, use T25 flask coated with 0.1% gelatin.

10. Add 8 mL retinal differentiation medium.
11. Centrifuge at 180 g for 3 min at 22–25°C.
12. Remove the supernatant, tap to loosen the cell pellet, and resuspend the cells in retinal differentiation medium.

Note: Amount of medium depends on the size of pellet. We resuspend 100 μL cell pellet in 10–12 mL differentiation medium.

13. Filter the cells with 40 μm cell strainer.
14. Count the number of cells by hemocytometer.

Note: Count the four quadrants at the four corners and divide the final number by four = ___ x 10^4 cells/mL for enhanced accuracy.

15. Adjust the concentration to 3–5 x 10^4 cells/mL with retinal differentiation medium.

Note: Most of the cell lines we differentiate show optimal differentiation efficiency at a density of 3 x 10^4 cells/mL (3000 cells in one well of 96-well plate). However, some cell lines require a different density. Depending on cell lines, optimization may be needed for optimal plating density.
16. Plate 100 μL cells into each well of a U-bottom ultra-low attachment 96-well plate. Pipet in reagent reservoir. Avoid bubbles by pipetting along wall of U-bottom 96-well plate.

17. Incubate the plate at 37°C under 5% O₂ and 5% CO₂ for 24 h.

△ CRITICAL: Day 0–7 are critical for differentiation. AVOID oxygen levels over 8%.

**Day 1. Addition of Matrigel**

⏱ TIMING: 2 h

18. Chill pipet tips, serological pipets, reagent reservoirs at −20°C for at least 30 min.

19. Add 2-ME to retinal differentiation medium to reach a final concentration of 55 μM and place on ice.

20. Thaw Matrigel aliquots on ice (at least 1 h).

Note: Depending on the stock concentration, each aliquot contains ~240 μL Matrigel for one 96-well plate.

21. After Matrigel is dissolved, add 900 μL cold retinal differentiation medium into each tube of Matrigel aliquots. Transfer 1140 μL Matrigel/media to the reservoir. Rinse the tube with another 900 μL cold retinal differentiation medium and transfer to the reservoir. Mix well with a chilled serological pipet.

22. Using chilled tips, add 20 μL diluted Matrigel to each well.

△ CRITICAL: When adding Matrigel, position the tips at the surface of media in each well. Do not put the tip too deep into wells to avoid disruption of embryoid bodies. Avoid adding Matrigel along the walls of each well as it may stick to the surface (Figure 1).

**Day 7. Transfer Organoids to Suspension Culture**

⏱ TIMING: 30 min–1 h

23. Pipet retinal organoids from a 96-well plate into a 15 mL tube using a 2 mL serological pipette. Transfer 12 wells at a time (Figure 2).

Alternatives: Cut open a P1000 tip and transfer the organoids using P1000 pipetman.
24. Let organoids settle to the bottom of the tube at 22–25°C and remove the media, leaving organoids in ~2 mL media.

Note: The time for organoids to settle depends on the amount of Matrigel taken during the transfer but it usually takes around 5 min. If the organoids do not settle, add 4 ml retina maturation medium (RMM I) supplemented with 55 μM 2-ME into the tube to dilute the Matrigel and facilitate this process.

25. Resuspend organoids in 10 mL 22–25°C retina maturation medium (RMM I) supplemented with 55 μM 2-ME.

26. Let organoids settle to the bottom of tube at 22–25°C (~2 min). Remove 10 mL media. Resuspend the organoids in 6 mL RMM I with 55 μM 2-ME and transfer to a 10 cm² polyHEMA-coated dish. Rinse the tube with another 6 mL of RMM I with 55 μM 2-ME and transfer to the same dish.

27. Incubate the dish at 37°C under 5% O₂ and 5% CO₂.

Day 10. Media Change for Retinal Organoids

© TIMING: 30 min

28. Reconstitute RMM I warmed to 22–25°C with 1 mM taurine, 20 ng/mL IGF1, 55 μM 2-ME, 26 μM DHA and 0.5 μM 9-cis retinal.

△ CRITICAL: Turn off the light of the hood to avoid isomerization of 9-cis retinal.
CRITICAL: Use the reconstituted RMM I as soon as possible (maximum 3 h).

29. Transfer all organoids to a 15-mL centrifuge tube with the 12 mL media using a 10 mL serological pipet (Figure 3).

Note: Position the tip of the serological pipet in the middle of the centrifuge tube and release the organoids slowly to avoid damage to tissue integrity.

30. While the organoids are settling down to the bottom at 22–25°C (~ 2 min), add 10 mL reconstituted RMM I to the dish.
31. Using a 2 mL serological pipet, position the tip to the bottom of the centrifuge tube and transfer the settled organoids back to the dish together with ~ 2 mL media. Gently swirl the dish (Figure 3).
32. Slightly tilt the dish, wait for 30 s for the organoids to settle and transfer ~10 mL media to a 15 mL centrifuge tube. Avoid picking up any organoid (Figure 3).
33. Add another 10 mL reconstituted RMM I to the dish.
34. Check the 15 mL centrifuge tube for organoids. Discard the tube if there are no organoids inside. Transfer the organoids to the dish as described above if there are any. Return the dish to 37°C under 5% O_2 and 5% CO_2.
35. Perform half-media change every alternate day.

Note: After D12, badly differentiated organoids can be removed, and sticking organoids can be separated under the microscope. We culture 10–16 organoids in 12–14 mL reconstituted RMM I in a 10 cm² polyHEMA-coated dish. Avoid putting too many organoids in a single plate.

Day 26. Media Change for Retinal Organoids

○ TIMING: 30 min

36. Reconstitute RMM II warmed to 22–25°C with 1 mM taurine, 20 ng/ml IGF1, 55 µM 2-ME, 200 ng/mL FGF1 and 0.5 µM 9-cis retinal.

△ CRITICAL: Turn off the light of the hood to avoid isomerization of 9-cis retinal.

△ CRITICAL: Use the reconstituted RMM II as soon as possible (maximum 3 h).

37. Slightly tilt the dish, remove ~10 mL media. Avoid taking any organoids.
38. Add 10 mL reconstituted RMM II.
39. Return the dish to 37°C under 5% O_2 and 5% CO_2.
Perform half-media change every other day.

EXPECTED OUTCOMES
As shown in previous publications (Chen et al., 2016, DiStefano et al., 2018, Brooks et al., 2019), embryoid bodies are formed at D1 as a homogenous sphere. Neuroepithelium can be observed by D4 as a bright rim on the organoids. Optic vesicles and optic cups appear at D7 and D10, respectively (Figure 4A). The mouse pluripotent stem cell lines we used include a transgene carrying GFP reporter under the control of the promoter of Nrl, which is the earliest specific marker for postmitotic rod photoreceptors; therefore, flow analysis is an efficient way to evaluate the biogenesis of rod photoreceptors in the resulting organoids (Figure 4B). GFP+ rod cells can be detected as early as D18 (0.5%–2%). Both the number of GFP+ cells and GFP fluorescent intensity increase as the organoids become more mature and the organoid cultures yield up to 80% GFP cells at D32 (60%–80%). Immunohistochemistry analyses consistently reveal rod photoreceptors as a dominant cell type.
type in retinal organoids (Figure 4C). In addition, biogenesis of cone photoreceptors, bipolar cells, Müller glia, horizontal and amacrine cells, and retinal ganglion cells can also be observed in organoid cultures.

LIMITATIONS

Our HIPRO protocol is modified from the serum-free culture of embryoid body-like aggregates with quick aggregation (SFEBq) protocol (Eiraku et al., 2011), and has been evaluated using 8 mouse pluripotent cell lines. The differentiation efficiency and endpoint maturity vary substantially by cell lines, frozen batches, and passages. In addition, although we managed to improve organoid cultures by manipulation of mis-regulated signaling pathway, photoreceptors in retinal organoids are not yet fully mature (Brooks et al., 2019). We note the need to modulate additional signaling pathways and incorporation of co-culture system with retinal pigment epithelium and/or bioengineering platform to facilitate further maturation of retinal organoids (DiStefano et al., 2018).

TROUBLESHOOTING

Problem: Low Differentiation Efficiency

Although the differentiation efficiency is greatly impacted by cell line, passage, batches of frozen stock, we were able to get more than 50% optic vesicle and optic cup formation using the eight mouse pluripotent stem cell lines we tested. Low differentiation efficiency may be the product of the quality of pluripotent stem cells, Matrigel concentration, and/or disruption of low oxygen environment.

Potential Solutions

1. Check the pluripotency of cells by immunostaining with pluripotency markers Oct4 and Nanog. The doubling time of healthy pluripotent stem cells should be 16–24 hours.
2. From D1 to D7, the oxygen level should not exceed 8%. Avoid frequent opening of hypoxia incubator or taking out differentiation plates from incubator for too long.
3. Check the concentration of Matrigel. Everything that touches Matrigel should be chilled to avoid solidification.
4. Use a different plating density from 3,000 cells to 5,000 cells per well of 96-well plate.

Problem: Endpoint Photoreceptor Maturity

Similar to differentiation efficiency, endpoint photoreceptor maturity can also be impacted by multiple factors. Furthermore, every organoid in a given batch of differentiation can vary dramatically, and therefore, not every neural retina developed an elongated axoneme and defined inner nuclear layer. Yet, we noted that approximately 50% of the organoids display the described mature morphology in over half of our cultures.

Potential Solutions

1. Change the lot of FBS. We observed lot-to-lot variation in endpoint maturity of photoreceptor differentiation.
2. Check the quality of 4% paraformaldehyde (PFA).

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AUTHOR CONTRIBUTIONS
Writing, H.Y.C. and R.A.K.; Editing, Supervision and Funding Acquisition, A.S.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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