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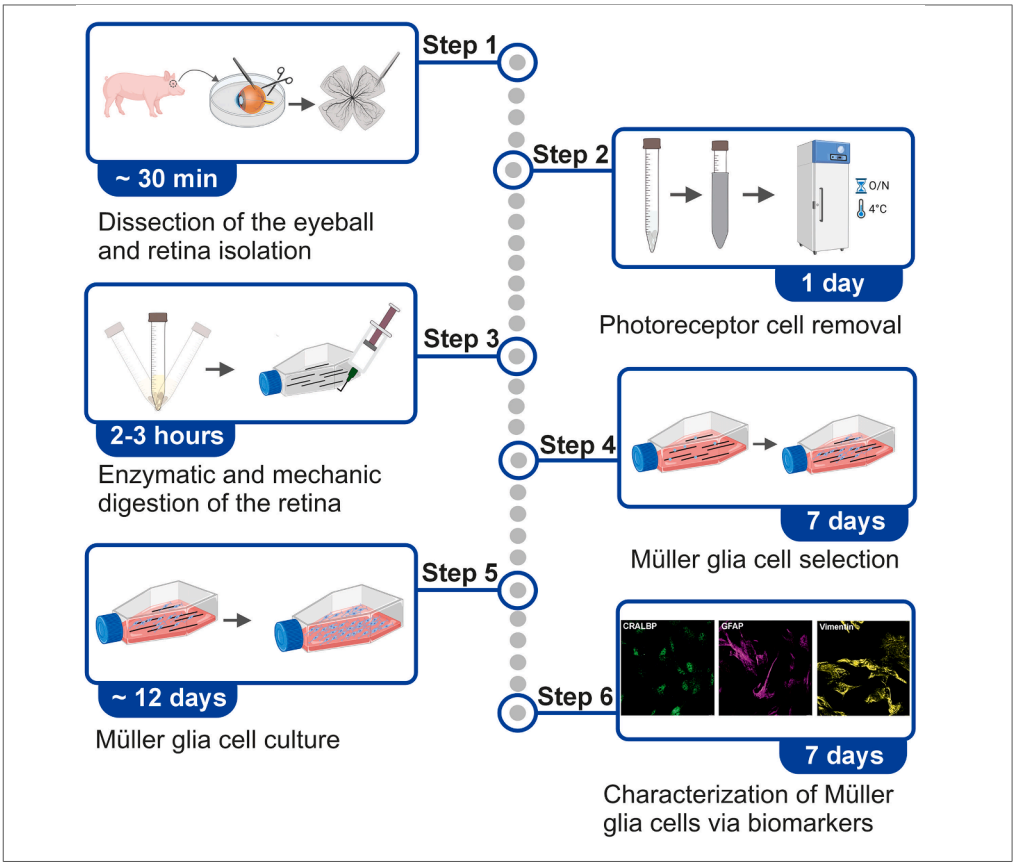
Protocol for the isolation, culturing, and evaluation of primary Müller glial cells from pig retina as cellular models in health and disease



Müller glial cells maintain retinal homeostasis and provide structural and metabolic support. The pig eye closely resembles the human eye in morphology and physiology, offering strong translational potential. Here, we present a protocol for isolating and culturing primary Müller glial cells from pig retina to study their function in health and disease. We describe steps for eyeball dissection, retinal isolation, photoreceptor cell depletion, and retinal digestion. We then detail procedures for the selection, culture, and characterization of Müller glial cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps for Müller glial cell isolation from porcine retina

Instructions for the selection of Müller glial cells via D-sorbitol media

Procedures for freezing and long-time storage of primary porcine Müller glial cells

Guidance on fluorescence microscopy analysis for primary porcine Müller glial cultures

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Protocol for the isolation, culturing, and evaluation of primary Müller glial cells from pig retina as cellular models in health and disease

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SUMMARY

Müller glial cells maintain retinal homeostasis and provide structural and metabolic support. The pig eye closely resembles the human eye in morphology and physiology, offering strong translational potential. Here, we present a protocol for isolating and culturing primary Müller glial cells from pig retina to study their function in health and disease. We describe steps for eyeball dissection, retinal isolation, photoreceptor cell depletion, and retinal digestion. We then detail procedures for the selection, culture, and characterization of Müller glial cells.

BEFORE YOU BEGIN

Müller glial cells are the principal glial cells of the vertebrate retina. Extending through the entire retina, they provide metabolic and structural support, maintain retinal homeostasis, and facilitate communication between all cell types in the retina.[1,2] They play a central role in the morphogenesis of the retina and are crucial for the development and manifestation of retinal diseases.[3] Their malfunctions promote degenerative processes.

Porcine models are getting increasingly important in translational vision research, as the anatomy and physiology of the pig eye closely resemble those of the human eye.[4] This makes them particularly valuable for studying disease mechanisms and evaluating therapeutic strategies for translation.[5,6] However, many cellular, molecular, and functional studies cannot be performed in vivo and remain restricted to cell cultures. To understand fundamental molecular processes in Müller glial cells that underlie retinal diseases, or evaluating drugs, primary Müller glial cells from animal models are excellent experimental cellular models for research.

The cultivation of primary Müller glial cells from humans and mice retinae is established.[7,8] However, there is currently no robust, standardized protocol for the cultivation and study of primary Müller glial cells from the pig eye. The protocol we describe here enables the isolation and long-term culturing and storage of primary Müller glial cells from mature retina of wild-type and retinal disease models.

After removal of the eyeball from the pig body all procedures should be performed under sterile conditions to avoid contamination and ensure reproducibility. Additionally, several chemicals and surgical instruments should be prepared before starting the experimental protocol (for detailed information please see key resources table and materials and equipment setup section).

Innovation

Here we describe a fast, cost-effective, and user-friendly protocol for the isolation and cultivation of primary Müller glia cells from the porcine retina. By combining elements of two previously established protocols for mouse and human retinae,[7,8] we developed a modified procedure that integrates two complementary Müller glia selection strategies. Specifically, we employed (i) a dark-cold incubation step to reduce neuronal contamination and (ii) a D-sorbitol based selection to achieve high culture purity, thereby eliminating the need for fluorescence-activated cell sorting (FACS). In addition, the new method enables the cryopreservation of Müller glial cells, allowing their use in later experiments at defined times and passage numbers. This is particularly advantageous working with large animal models, like porcine models as their numbers are usually limited and it is therefore often difficult to obtain samples, especially from age-matched individuals at the same time.

Institutional permissions

All experimental procedures involving animal tissues should be approved by the appropriate institutional ethics committee and conducted in accordance with relevant national and institutional guidelines and regulatory standards. Researchers intending to reproduce these experiments must obtain approval and necessary permissions from their respective institutional and regulatory authorities before initiating the work.

For this study the work on pigs has been conducted under the supervision of the responsible regulatory authorities: the Regierung von Oberbayern at LMU Munich (file numbers ROB-55.2-2532.Vet_02-19-195 and ROB-55.2-2532.Vet_02-18-134) and the State Veterinary Administration of the Czech Republic IAPG Libechov approved animal experiments under the experimental protocol number 75/2019.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-GFAP (Glial fibrillary acidic protein)	Sigma Aldrich	Cat#G3839
Mouse monoclonal anti-Vimentin	Santa Cruz	Cat#SC-32322
Guinea pig polyclonal anti-MAP2 (Microtubule associated protein 2)	Synaptic systems	Cat#188004
Rabbit polyclonal anti-CRALBP (Cellular retinaldehyde- binding protein)	Proteintech	Cat#15356
Alexa Fluor 488–conjugated donkey anti-rabbit IgG	Invitrogen	Cat#A-21206
Alexa Fluor 647–conjugated donkey anti-mouse IgG	Invitrogen	Cat#A-31571
Alexa Fluor 488–conjugated donkey anti-guineapig IgG	Invitrogen	Cat#A-21202
Chemicals and reagents		
Iodine solution	Betaisodona	N/A
DMEM + GlutaMAX	Thermo Fischer Scientific	Cat#10569010
DMEM without D-glucose and sodium pyruvate	Thermo Fischer Scientific	Cat#11966025
Amphotericin B	Sigma Aldrich	Cat#1397-89-3
Endothelial growth factor (EGF)	Sigma Aldrich	Cat#SRP3027
Fetal bovine serum (FBS)	Cytiva	Cat#SV30160.03
TrypLE™ Express (recombinant proteases in defined solution)	Thermo Fischer Scientific	Cat# 12604013
D-sorbitol	Sigma Aldrich	Cat#S1876
Penicillin/Streptomycine	Thermo Fischer Scientific	Cat#15140122

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
1N Sodium hydroxide (NaOH) solution	Sigma Aldrich	Cat#1.09137
1N Hydrocloric acid solution	Sigma Aldrich	Cat#H1758
DPBS (no calcium, no magnesium)	Gibco	Cat#14190094
Poly-L-lysine	Sigma Aldrich	Cat#P4707
Trypsin-EDTA (0.05 %)	Thermo Fischer Scientific	Cat#25300054
PFA (paraformaldehayde)	Sigma Aldrich	Cat#30525-89-4
DMSO	Sigma Aldrich	Cat#67-68-5
Donkey serum	Abcam	Cat#ab7475
Bovine serum albumin	Carl Roth GmbH +Co. KG	Cat#8076.2
Triton-X-100	Sigma Aldrich	Cat#102533092
Mowiol	Carl Roth GmbH +Co. KG	Cat# 0713.1
DABCO (anti-bleaching agent)	Carl Roth GmbH +Co. KG	Cat#0718.2
4′,6-Diamidino-2-Phenylindole (DAPI)	Carl Roth GmbH +Co. KG	Cat#6335.1
TRITC (Rhodamine)- coupled Phalloidin	Sigma Aldrich	Cat#P1951
Experimental models: organisms/strains		
Domestic pig: *Sus scrofa domesticus, wild-type, 7-months-old, male and female*	LMU, PIGMOD	N/A
Humanized *USH1C*^{R31*} knock in, domestic pig *Sus scrofa, homozygous, 7-months-old, male and female*	Grotz et al., 2022[5]	N/A
Software and algorithms		
ImageJ software	National Institutes of Health	http://imagej.nih.gov/ij/download.html
GraphPad Prism 8	GraphPad Software	https://www.graphpad.com/
NC-View™ Software	Chemometec	https://chemometec.com/software/nc-view/
Other		
Inverted fluorescence light microscope	Leica	Cat#16341
Upright motorized fluorescence microscope	Leica	Cat#DM6000B
CO_2 cell culture incubator	Eppendorf	Cat#6734000041
Tabletop centrifuge	NEO-Lab table centrifuge	Cat# D6020
Medium-sized scissors	A.dumont & Fils	Cat#T5074
Dumont #3 curved forceps	A.dumont & Fils	Cat#T504
Dumont #4 standard tip forceps	A.dumont & Fils	Cat#T505
Kelly hemostatic forceps	Sigma Aldrich	Cat#Z168874-1EA
Automated cell counter	Chemometec	Cat#NC-202
Filter papers	Carl Roth GmbH +Co. KG	Cat#A126.1
Freezing container	Thermo Fischer Scientific	Cat#5100-0001
Cryovials	Sarstedt AG & Co.	Cat#72.379
Via2-Casette™	Chemometec	Cat# 941-0024
100 mm^2 petri dish (cell culture)	Greiner	Cat#664160
T-75 culture flasks	Greiner	Cat#658170
6-well culture plates	Greiner	Cat#657160
18G needle	Braun	Cat#4665120B
Sterile gauze pads	Param GmbH	Cat#03856121
50 mL Centrifuge Tubes	Sarstedt AG & Co	Cat# 62.547.254
15 mL Centrifuge Tubes	Sarstedt AG & Co.	Cat#62.554.502
0.22 µm filter	Carl Roth GmbH +Co. KG	Cat#KH541

MATERIALS AND EQUIPMENT

Starvation medium

Reagent	Final concentration	Amount
DMEM + GlutaMAX	1×	495 mL
Penicillin/Streptomycine	1× (1%)	5 mL
Total	**N/A**	**500 mL**

After preparation of starvation medium, store it at 4°C and use it within 6 months.

Selection medium		
Reagent	Final concentration	Amount
DMEM without D-glucose and sodium pyruvate	1×	39 mL
1M D-sorbitol	25 mM	1 mL
Fetal bovine serum (FBS)	10%	4 mL
Penicillin/Streptomycine	1× (0.5%)	200 µL
Amphotericin B	1× (0.5%)	200 µL
EGF	100 ng/mL	3.6 µL
Total	**N/A**	**44.76 mL**

After preparation of the selection medium, store it at 4°C and use it within 1 week.

Complete medium		
Reagent	Final concentration	Amount
DMEM + GlutaMAX	1×	450 mL
Fetal bovine serum	10%	45 mL
Penicillin/Streptomycine	1× (1%)	5 mL
Total	**N/A**	**505 mL**

After preparation of the complete medium, store it at 4°C and use it within 1 month.

Stock formaldehyde solutionReagent	Final concentration	Amount
Paraformaldehyde (PFA)	15%	15 g
DPBS (no calcium, no magnesium)	N/A	100 mL
1N Sodium hydroxide (NaOH) solution	N/A	~5-6 drops
1N Hydrochloric acid (HCl) solution	N/A	~5-6 drops
Total	**N/A**	**100 mL**

After preparation of the 15% stock formaldehyde solution, store it at −20°C and use it within 6 months.
Prepare the fixation solution freshly and do not freeze.

⚠ CRITICAL: Multiple freeze and thaw steps can reduce the fixation efficiency.

Freezing medium		
Reagent	Final concentration	Amount
Complete media	90%	900 µL
DMSO	10%	100 µL
Total	**N/A**	**1000 µL**

Prepare fresh freezing medium just before use and keep it on ice until needed.

TritonX-100 solution		
Reagent	Final concentration	Amount
0.5% TritonX-100	0.1%	200 µL
DPBS (no calcium, no magnesium)	N/A	800 µL
Total	**N/A**	**1000 µL**

After preparing the 0.1% Triton X-100 solution, store it at −20°C for long-term use or at 4°C if it will be used within one week.

Blocking solution		
Reagent	Final concentration	Amount
BSA	2%	0.1 g
0.5 mL Donkey Serum	10%	0.5 mL
DPBS (no calcium, no magnesium)	N/A	5 mL
Total	**N/A**	**5.5 mL**

Always prepare the blocking solution fresh. Any remaining solution can be stored at 4°C and used within one week.

⚠ CRITICAL: Do not freeze the blocking solution.

STEP-BY-STEP METHOD DETAILS

Enucleation, dissection of the eyeball, and isolation of the pig retina (day 0)

Timing: 30–40 min

Note: Animals are sacrificed in deep lethal anesthesia by an authorized experienced scientist or veterinarian. Subsequently, eyeballs are surgically removed (enucleation) and placed into 50 mL tubes labeled with the content: animal number, "left" or "right" eye. For the transfer to the laboratory tubes are kept on ice.

Note: For preliminary test runs of the protocol fresh eyes can be obtained from a local slaughterhouse.

⚠ CRITICAL: Before starting, ensure that you have access to freshly isolated porcine eyes, ideally processed within 2 h post-mortem to prevent retinal degradation.

1. Remove muscle and fat tissue (Figure 1A) from the enucleated eyeball by using sharp medium-sized Dumont scissors.
2. Before starting the dissection, gently hold the eyeball with a sterile forceps Dumont#4 and Dumont#3.
   a. Dip 3 times each into the sterile DPBS solution and iodine solution (100 mg/mL) to prevent bacterial contamination.
   b. Then three times again in fresh DPBS solution for washing away the iodine, indicating brownish color of the sclera (Figure 1B).

⚠ CRITICAL: Perform step 3 under the laminar flow hood while strictly maintaining aseptic conditions. After this step, all procedures must be carried out under sterile conditions.

3. Place the eyeball onto the sterile gauze pad and hold it gently (Figures 1A and 1B). Avoid applying excessive pressure to the eyeball, as this can result in retinal damage or detachment.
4. To prepare the eyecup, use a sterile scalpel to make a small incision at the junction between the iris and sclera (arrow) (Figure 1B).
   a. Starting from this incision, carefully cut along the red dotted line (Figure 1B) using curved scissors.
   b. Remove the cornea and lens from the eyeball using a forceps (Dumont#3 and Dumont#4) (Figure 1B).
5. Gently squeeze the eyecup to expel the vitreous humor (Figure 1C).
6. Using forceps (Dumont#3 and Dumont#4) and medium-sized Dumont scissors, make cuts in the eyecup to prepare a flat mount (Figure 1D).

Note: The radial relaxing cuts relieve mechanical tension on the retina, and the retina does not curl and fold before isolation. This increases both yield and quality of the isolated Müller glia cells.

7. Use the back curved forceps (Dumont#4) to carefully peel off the neural retina from the underlying retinal pigment epithelium (RPE) and sclera.

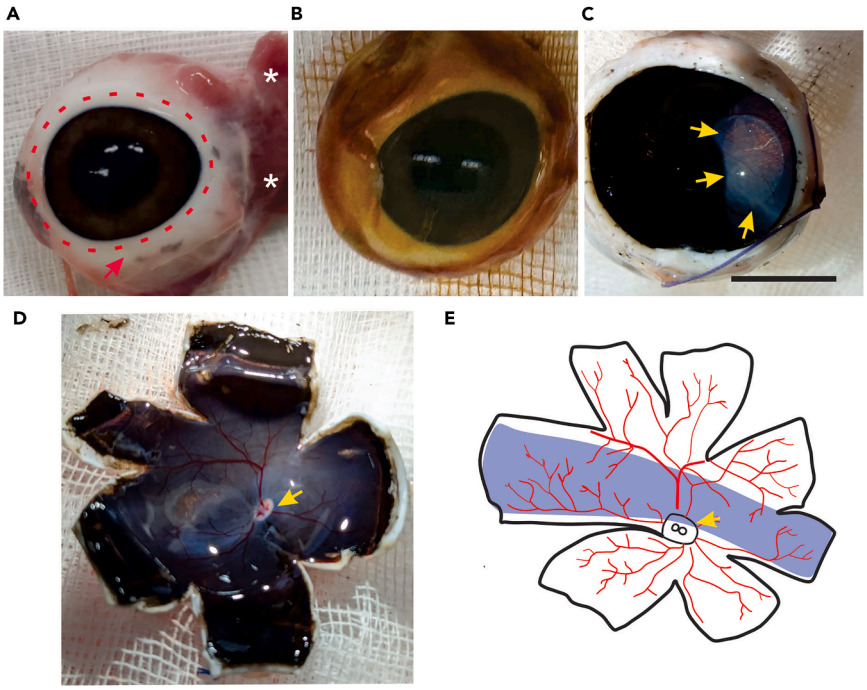

Figure 1. Enucleation of the porcine eye and eyeball dissection

(A) Porcine eyeball on sterile gauze pad, asterisks indicating muscles which must be removed. The red dashed line shows the incision line for the eye cup preparation. Red arrowhead indicates the small incision site.

(B) Excessive muscles removed from the eyeball and sterilized with brownish iodine/DPBS solution.

(C) DPBS washed eye cup after removing lens and vitreous humor in Steps 4-5. Arrowheads indicate the whitish retina in the back of the eyecup.

(D) Flat mounted porcine eye cup by incisures, ready for isolation of the retina. Porcine eye flat mounted by scissors cuts, prepared for the isolation of the neuronal retina. Yellow arrowhead indicates optic nerve head the flat mounted porcine eye. Scale bar: 1 cm.

(E) Flat mounted retina scheme, belongs to left eye. Blue highlighted part shows cone photoreceptor-rich region called visual streak. Yellow arrowhead indicates optic nerve head.

⚠ CRITICAL: As the retina and RPE are attached to each other, carefully peel off the neural retina to avoid non-retinal cell contamination in the culture.

Note: The neural retina remains attached at the optic disc, where the optic nerve exits the eye. After peripheral detachment, carefully dissect this remaining attachment with fine scissors and/or forceps to completely detach and peel off the retina as an intact layer. In addition, visual inspection of the isolated retina with the naked eye and/or under a stereomicroscope is important to ensure that there are no dark-pigmented melanomas in the isolated retinal tissue, which would indicate contamination with RPE cells.

Photoreceptor cell removal from the retina (day 0)

Timing: 1 day

8. Place isolated retina in a 15 mL tube containing starvation medium, store it in the dark at 4°C for 14-16 h.

⚠ CRITICAL: To protect the retina from light, wrap the tube with aluminum foil for step 8.

Note: Be sure to add penicillin and streptomycin to the starvation medium.

Note: In the dark rod photoreceptors require high energy to maintain their resting membrane potential[9]; therefore, the majority of photoreceptors will die during 14–16 h incubation in the glucose-free starvation medium in the dark.

Note: After 14–16 h of incubation under starvation conditions in the dark, most rods are expected to die first, followed by the cones which will survive longer but also perish during the 14–16 h of incubation in starvation medium.

Enzymatic and mechanical digestion of the retina (day 1)

Timing: 2–3 h

9. Pre-warm the TrypLE™ digestion solution and selection medium in a 37°C water bath prior to the next steps.

Note: For gentle dissociation, the use of recombinant trypsin TrypLE™ is recommended instead of trypsin, purified from pancreas, or papain often used in other protocols, as these can negatively affecting the isolation of Müller glial cells from pig retinae.

10. Enzymatic digestion of the porcine retina:
    a. Transfer the retinal tissue with sterile forceps into a new sterile flat-bottom tube containing 5 ml of pre-warmed TrypLE™ digestion solution.

Note: Do not a pipette to transfer retina by suctioning. Use fine forceps for controlled mechanical handling.

    b. For tissue digestion place the tube in a 5% CO_2 incubator at 37°C for 60 min, inverting it every 20 min during digestion.
11. Using sterile forceps, transfer the digested retinal tissue into a 60 mm cell culture dish containing 10 ml of complete medium.

Note: Do not use a pipette to transfer retina by suctioning. Use fine forceps for controlled mechanical handling

12. Mechanical digestion of the porcine retina:
    a. Cut the tissue into ~1 × 1 mm pieces using sterile scissors and forceps (Dumont medium-sized scissors and Dumont#4) in complete medium.
    b. Transfer the retinal pieces into T75 cell culture flasks using sterilized glass Pasteur pipettes.

Note: When transferring the retinal pieces into T75 cell culture flasks, try to collect only the retina pieces and avoid taking up complete medium as much as possible.

Note: The total retina from one eyeball can be divided into three to four T75 cell culture flasks.

    c. Bend a sterile 18 G needle to a 90° angle using hemostatic forceps. Using the angled needle, gently separate the dissected retinal pieces and distribute them evenly across the bottom of the flask to prevent clumping.
    d. Firmly press and scratch the retinal pieces on the bottom of the T75 flasks by using an 18 G angled needle tip ensuring they adhere to the flask surface.

Note: This enhances the success rate of primary porcine Müller glia cell culture. Once all pieces are firmly fixed, position the flask vertically and gently add 7 mL of selection medium.

Note: Take care of the medium while you are adding to the flask not to dislodge the attached retinal pieces.

Selection of Müller glial cells via D-sorbitol media (day 1)

Timing: 7 days

These steps will be used for the separation of primary Müller glial cells from other retinal cell types by using selection medium. This medium contains D-sorbitol which is a selective sugar for the isolation of Müller glia cell.[8]

⚠ CRITICAL: Always prepare the selection medium with a fresh D-sorbitol solution. D-sorbitol is a sugar and is therefore susceptible to contamination.

Note: Be sure to add penicillin and streptomycin antibiotics together with amphotericin B as an antifungal agent to the selection medium.

13. Place the flask vertically at 37°C with 5% CO_2 in the incubator for 15 min to promote better attachment of the retinal pieces to the bottom of the T75 flask.
14. Place the T75 flask horizontally. The 7 ml of medium is sufficient to cover the bottom of the flask and helps minimize tissue detachment during culture.
15. Culture the cells for 1 week at 37°C with 5% CO_2 in the incubator.

⚠ CRITICAL: Do not disturb the cells for 7 days to promote optimal attachment to the T75 flask.

Note: Check one flask after 24 h to confirm successful cell attachment (Figure 2A).

Primary Müller glial cell culture (day 7)

Timing: 10–12 days

At this stage, the selection of Müller glia cells using selection medium is completed, and conditions are adjusted to promote cell growth (Figure 2B).

16. Add another 7 mL complete medium to the T75 flask.

⚠ CRITICAL: Be careful when adding medium. Gently dispense it along the wall of the flask rather than directly onto the cells, as some cells may still be loosely attached.

Note: Be sure to add penicillin and streptomycin to the complete medium.

17. After 2 days, remove the entire medium and carefully add 14 mL of fresh complete medium to the cells.
18. Replace the old medium with fresh complete medium every 2 days.

Note: Primary porcine Müller glia cells can be cultivated up to 2 weeks.

Freezing and long-time storage of primary porcine Müller glial cells (day 19)

On Day-19, primary porcine Müller glia cells can be frozen and stored for further experiments to maintain a lower passage number.

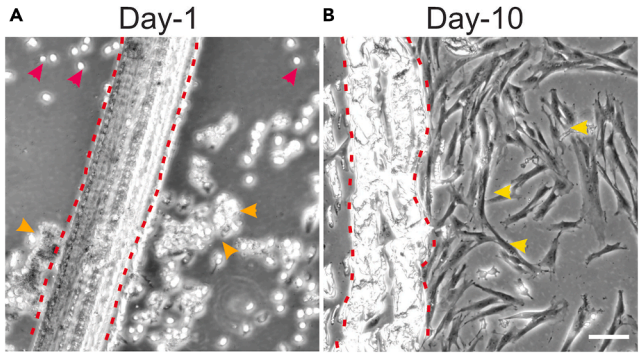

Figure 2. Expansion of the Müller glia cells in the culture

(A) Bright field microscopy image showing mechanically dissociated retinal cells: individual round cells, loosely attached to culture dish after the needle-scratch (magenta arrowheads) and cell accumulations or tissue fragments (orange arrowheads) adhering to the needle-scratch (red dashed-lines) on Day-1 of Müller glia cell culture in selection medium.

(B) Upon completion of the Müller glia cell selection phase and after three days in complete medium (after Step 18, Day-10), primary Müller glia cells (yellow arrowheads) proliferate and extend from the needle-scratch (red dashed-lines) within the culture flask. Scale bar: 250 μm.

19. Warm the complete medium, DPBS and trypsin up to 25°C.
20. Prepare the desired number of cryovials approximately 3 vials per T75 flask and freezing container filled with isopropanol.
21. Prepare the freezing medium that contains 90% complete medium and 10% of DMSO.
22. Take the T75 flask out from the incubator.
23. Discard the medium and gently wash the cells with 10 mL of DPBS.
24. Discard the DPBS and add 2 mL of trypsin onto the cells.
25. Incubate the flask at 5% CO_2 incubator for 9-10 min.
26. Take the flask out and examine the cells under the microscope to confirm the cell detachment.
27. Add 10 mL of complete medium to the cells and pipette up and down 12 to 13 times with serological pipette to ensure thorough dissociation of cell clumps and obtain a single-cell suspension.
28. Collect the whole cell suspension and place into the 15 mL tube.
29. Centrifuge the cells at 0.3 × *g* for 5 min at 25°C.
30. Discard the supernatant and resuspend the cell pellet with 3.5 mL of freezing medium.
31. Count the cell number:
    a. Pipette 100 μL of the resuspended cell suspension.
    b. Load the sample into the Via2-Casette™ for automated cell counting.
    c. Record the number of live cells reported by the cell counter by NC-View™ software.
    d. Based on the live cell count, calculate the required volume of cell suspension for 3×10^6 cells per cryovial.
32. Distribute the cell suspension into the cryovials.
33. Freeze the cryovials within the freezing container at −80°C for 14–16 h. Next day place your samples in the −80°C freezer or for long-term storage transfer the cryovials to the liquid nitrogen tank.

Indirect immunofluorescence staining of the primary porcine Müller glial cells for molecular markers (day 19)

Timing: 7 days

In the following steps, we describe how to characterize primary porcine Müller glia cells by immunocytochemistry applying specific cell markers. In each experiment, at least three areas are randomly

selected in at least three cover glasses and imaged using the 40x objective lens of the Leica DM6000B fluorescence microscope (Leica Microsystems, Bensheim, Germany).

34. First, place five of the sterile 10 mm round coverslips to one well of the 6-well plate.

⚠ CRITICAL: Place the coverslips like a flower shape: one to the middle and four to the surrounding of the middle one. This arrangement helps to keep the coverslips stable and properly positioned within the well during cell culture.

35. Coat the coverslips with 500 µl of 0.01% sterile-filtered Poly-L-lysine and wait 1 to 2 min.
36. Remove the poly-L-Lysine solution and place the 6-well plate on the 37°C incubator for 30 min in order to dry it.

Note: The sterile poly-L-Lysine solution can be used up to 5 times, therefore discarded poly-L-Lysine can be stored at 4°C for further usage.

37. Wash confluent primary porcine Müller glia cells in the T75 flask with DPBS, then add 2 mL of 0.05% trypsin and incubate at 37°C for 5 min to detach the cells.
38. To neutralize the trypsin and collect the cells, add 8 mL of complete medium to the T75 flask, then transfer the cell suspension into a 15 mL centrifuge tube.
39. Centrifuge the cell suspension in a tabletop centrifuge at 0.3 × *g* at 25°C for 5 min to collect the cell pellet.
40. Carefully discard the supernatant and resuspend the cell pellet in 5 mL of complete medium.
41. Count the number of cells.
    a. Pipette 100 µL of the resuspended cell suspension.
    b. Load the sample into the Via2-Casette™ for automated cell counting.
    c. Record the number of live cells reported by the cell counter by NC-View™ software.
    d. Based on the live cell count, calculate the required volume of cell suspension for your experiment.
42. Seed 500,000 cells onto the well containing 5 coverslips and incubate for 3 days at 37°C with 5% CO_2 in the incubator.
43. On the following day after cell seeding, exchange the medium with 2 mL of fresh complete medium.
44. After 3 days of incubation, aspirate the medium and gently wash the coverslips 2 times with 70 µL of DPBS.
45. Prepare a wet chamber to maintain humidity during incubation. To prepare the wet chamber:
    a. Label the 100 mm^2 petri dish for the experiment.
    b. Cut the filter paper to the size of the petri dish so that it fits neatly on the bottom.
    c. Moisten the filter paper with water after placed onto the petri dish and remove the excess water.
    d. Cut a piece of parafilm sheet to place onto the filter paper.
46. Remove the coverslips from the well and place them onto the parafilm sheet.
47. Fix the cells by applying 70 µL of fixation solution to each coverslip and incubate for 10 min at 25°C.
48. Wash the cells with 70 µL of DPBS to remove any residual fixation solution.
49. Permeabilize the cells with 70 µL of Triton-X-100 solution for 10 min 25°C.
50. Wash the cells twice quickly with 70 µL of DPBS.
51. Incubate the cells with 70 µL of blocking solution (10% donkey serum and 1% bovine serum albumin in DPBS) for 90 min in 25°C.
52. After blocking, incubate the cells with 50 µL of primary antibodies for 12–16 h at 4°C.

Note: We used rabbit anti-CRALBP (1:200 dilution), mouse anti-GFAP (1:500 dilution) and mouse anti-Vimentin (1:200 dilution), guineapig anti-MAP2 (1:500 dilution) antibodies diluted in blocking solution.

Note: Prepare all the primary antibody dilutions within the blocking solution and keep them on ice.

53. After primary antibody incubation, wash the cells three times quickly with 70 μL of DPBS.
54. Apply 50 μL of secondary staining mix solution to each coverslip.
    a. Prepare the secondary staining mix solution in a 1.5 mL tube.
    b. Incubate the coverslips at 25°C for 90 min in a humidified chamber.

Note: The secondary staining mix solution should contain the following components, all diluted 1:400 in blocking solution: Alexa Fluor 488–conjugated donkey anti-rabbit IgG, Alexa Fluor 647–conjugated donkey anti-mouse IgG, Alexa Fluor 488–conjugated donkey anti-guineapig IgG and dyes 4′,6-Diamidino-2-Phenylindole (DAPI) for nuclear DNA staining and TRITC-Phalloidin for labeling actin filaments (F-actin).

⚠ CRITICAL: Secondary antibodies and dyes should be centrifuged by tabletop centrifuge for 30 s before usage to remove aggregates that may cause nonspecific fluorescence signals.

Note: Prepare all the secondary antibody and dye dilutions within DPBS as 1:400 working dilution and keep them on ice.

55. Wash the cells with 70 μL of DPBS two times and ddH_2O one time rapidly.
56. Mount the coverslips in Mowiol containing 0.1% DABCO (anti-bleaching agent) on labeled microscopy slides.

⚠ CRITICAL: Perform the steps 54-56 under low-light conditions to protect the fluorophores from bleaching and preserve fluorescence signal intensity.

Note: In the next steps, we demonstrate how to assess the purity of primary porcine Müller glia cultures using well-established Müller glia cell and neuron markers for immunostaining and validated secondary antibodies suitable for Müller glia cells. A total number of ~150 cells should be counted for the quantification.

Fluorescence microscopy and image processing for purity of primary porcine Müller glial cell cultures (day 19)

Note: Slides of immunostained primary porcine Müller glial cells are analyzed for anti-MAP2, anti-Vimentin and DAPI using a Leica DM6000B fluorescence microscope.

57. Open the images using ImageJ software.
58. To generate z-projection:
    a. Go to "Image - Stacks - Z Project - Maximum Projection".
    b. Save the processed digital image in a previously created folder on a computer or in the cloud.

⚠ CRITICAL: If z-projection is not created, only a single focal plane will be visible, which may not represent the entire cellular structure.

59. Select an exported and processed image from the 'image' folder.
60. Split the merged image.

a. Go to "Image - Color - Split channels".
b. Pick the channels which contain DAPI and anti-Vimentin staining.

61. Click to "multi-point selection" tool.
    a. First, count all DAPI positive cell nuclei with the tool.
    b. Second, count all Vimentin positive (Vim+) cells.
62. Generate an Excel sheet for the measurements.
63. Define the total DAPI positive nuclei (DAPI+) numbers as 100%.
64. Calculate the percentage of the Vim+ cell numbers relative to the DAPI+ cell numbers.

Note: Experimental controls stained without secondary antibodies should show no fluorescence signal, suggesting that the observed fluorescence detected specifically from the primary-secondary antibody interaction.

Light microscopy and image processing for phenotypic comparison of primary porcine Müller glial cells

Note: For the longitudinal cell length measurement, light microscope is used for the primary porcine Müller glia cell imaging. After images are acquired, they are processed by using ImageJ software for the longitudinal cell length measurement.

65. Open the images using ImageJ software.
66. Select an image from the 'image' folder.
67. Click to "Segmented line" tool.
    a. Define the cell with segmented line.
    b. Right click to finish the line.
    c. Measure the line and it will give you the results on the results table as "length".
    d. Measure all cells on the image.
68. Generate an Excel sheet for the measurements.
69. Export the data from excel sheet to GraphPad Prism software for the statistical analysis.

Note: For the Müller glia cell area measurement, slides of stained primary porcine Müller glial cells are analyzed for TRITC Phalloidin, anti-Vimentin and DAPI using a Leica DM6000B fluorescence microscope.

70. Repeat the steps between Steps57–60a.
71. Pick the channel containing TRITC Phalloidin (F-actin) staining for the area measurement.
72. Crop and duplicate the region of interest of image.
73. Set the scale.
    a. If the image has a scale bar "Analyze – Set Scale".
    b. Draw a line over the scale bar, enter the known distance and units and click "OK".
74. Convert the image to 8-bit image "Image – Type – 8-bit".
75. Tick the desired area measurement "Analyze – Set Measurements - Area".
76. Threshold the image "Image – Adjust – Threshold - Otsu" and click "Apply".
77. Generate a binary image "Process – Binary – Make Binary".
78. To get a smoothened image "Process – Binary – Fill Holes".
79. To create the selection "Edit – Selection – Create Selection".
80. Put the selection to the ROI manager "Analyze – Tools – ROI Manager – Add".
81. Open the duplicated image and click to "Add". Now, region of interest for area measurement is selected.
82. Go to Analyze – Measure and you will get the data for area measurement under the column "Area".
83. Generate an Excel sheet for the measurements.
84. Export the data from excel sheet to GraphPad Prism software for the statistical analysis.

EXPECTED OUTCOMES

In this protocol, we describe a cost-efficient and reproducible method for the isolation and cultivation of primary Müller glia cells from porcine retinae, without the need for commercial kits or specialized equipment. Using this approach, a healthy and easy-to-maintain primary Müller glia culture can be established from both healthy wildtype porcine retinae and retinae derived from porcine disease models. Our data show that primary Müller glial cell cultures obtained using this protocol are highly pure and well suited for studying cell morphology and physiological processes specific to Müller glial cells. In addition, primary Müller glia cell cultures are well qualified for translational approaches such as the evaluation of therapies or toxicity and drug testing. Long-term storage of cultures also allows for planning and conducting experiments at short notice without the need to consider complex breeding programs for experimental pigs. Finally, working with primary cell cultures enables basic cell biology research and translational ventures without the use of laboratory animals, leading to a widely welcomed reduction in the number of laboratory animals employed.

Assessment of the primary porcine Müller glial cell culture purity

The isolated primary Müller glia cells can be identified by fluorescence microscopy using established Müller glia cell markers, namely antibodies against glial fibrillary acidic protein (GFAP), cellular retinaldehyde-binding protein (CRALBP), and Vimentin (Figure 3). To confirm the purity of the cultures, we also performed immunofluorescence co-staining for microtubule-associated protein 2 (MAP2), an established marker for primary retinal neurons. The absence of anti-MAP2 immunofluorescence in the primary Müller glia cell cultures (Figure 4) indicates that the culture is a pure culture and does not contain any neurons.

For quantitative analysis, we counted DAPI-positive (DAPI+) and vimentin-positive (Vim+) cells in the culture and calculated their ratio to determine the percentage of Vim+ cells representing purity of the culture (Figure 5).

Phenotypic comparison of primary Müller glial cells in/under pathological and healthy conditions

The present protocol can also be used to analyze putative phenotypes in Müller glia cells of pig models for retinal diseases. Here we analyzed morphological and physiological phenotypic changes in primary Müller glia cells comparing cells isolated from healthy porcine retinae and retinae of the humanized USH1C^{R31*} pig.[5] This pig model is the first established porcine model for the human Usher syndrome (USH), a clinical and genetically complex autosomal recessive disease. The most severe subtype USH1 is characterized by profound deafness, vestibular dysfunctions, and vision loss.[10,11]

The USH1C^{R31*} was generated by the exchange of the 2nd exon of the porcine *Ush1c* gene by the human *USH1C* of a USH1C patient carrying the pathogenic USH1C^{R31*} mutation. This model is currently used in ongoing research to investigate the mechanisms that lead to USH1 and to evaluate the efficacy of preclinical therapeutic strategies for USH1C. There is currently growing evidence that *USH1C* mRNA and the harmonin protein is expressed to a high degree, if not exclusively, in retinal Müller glial cells[12–14] and that the primary cause of USH1 disease therefore may originate in Müller glial cells. Here, we analyze the morphological phenotypes of primary Müller glial cells from pigs derived from USH1C^{R31*} pigs compared to wild-type conditions. To this end, we measured cell lengths and the area occupied by the cells (Figure 6). These morphometric analyses reveal that primary Müller glial cells from USH1C^{R31*} pigs are significantly longer and occupy less cell area compared to healthy primary Müller glial cells from pigs.

LIMITATIONS

A major limitation of this protocol is that primary Müller glial cells in culture do not fully reflect the morphological and physiological properties of Müller glial cells in the retina in vitro. Cultured

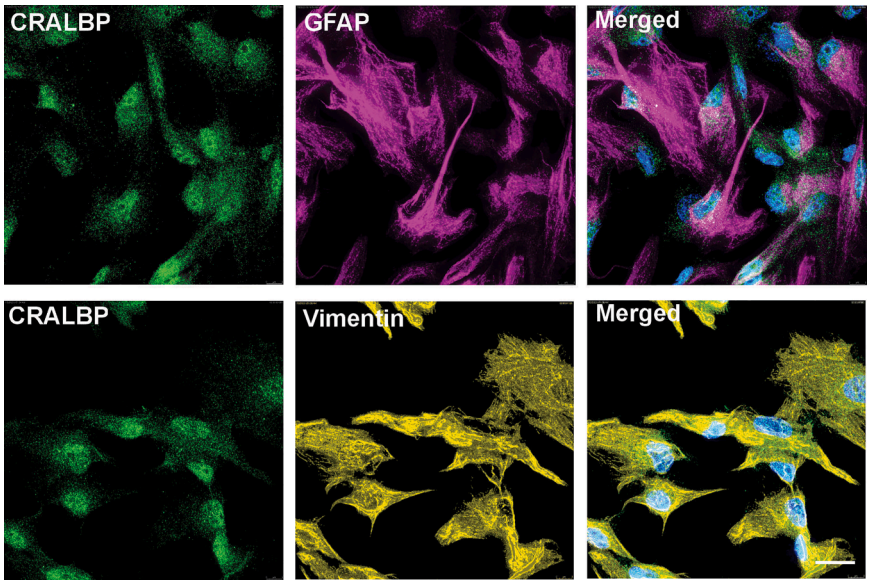

Figure 3. Characterization of wild-type primary porcine Müller glia cells

Indirect immunofluorescence microscopy of primary porcine Müller glia cells stained with antibodies against CRALBP (green), GFAP (magenta), and Vimentin (yellow) to confirm Müller glia cell identity. Nuclear DNA was counterstained with DAPI (blue).

Indirect immunofluorescence confirms that the cultured cells express Müller glial markers, verifying their identity through established biomarkers. Cells are isolated from 7-months-old wild-type porcine retina, passage number 3. Scale bar: 20 μm.

isolated primary Müller glial cells lack the structural and molecular interactions, molecular exchange processes, and reciprocal signalling with neighbouring retinal cells, particularly with photoreceptor cells and other retinal neurons. These cell-cell communications dynamically modulate morphology, physiology, and function of Müller glial cells in vivo. This limitation should be carefully considered when designing experiments with isolated primary Müller glial cell cultures and expectations for the outcome. In addition, we like to highlight that before working with experimental animal samples, we recommend practicing the procedure on pig eyes obtained from slaughterhouses to familiarize yourself with handling porcine eyes and retinal tissue. Working with age-matched pigs can be challenging, as obtaining sufficient numbers of both healthy and diseased samples is often difficult. Therefore, experiments should be planned carefully in advance to maximize the use of available samples and to ensure statistically relevant results.

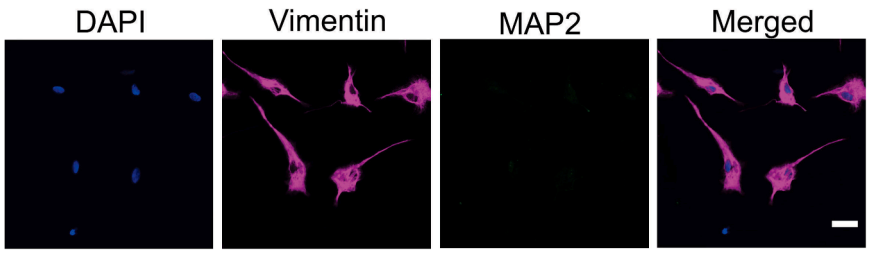

Figure 4. Exclusion of neuronal cell contamination in primary Müller glia cell cultures

Immunofluorescence analysis of porcine retina-derived primary Müller glia cell cultures double stained for the neuron marker microtubule-associated protein-2 (MAP2) and Müller glia marker vimentin (magenta).

DAPI (blue) indicates nuclei. Staining shows that the cultured cells exhibited no MAP2 signal, confirming the absence of neuronal contamination in the primary porcine Müller glial cell cultures.

Cells are isolated from 7-months-old wild-type porcine retina, passage number 3. Scale bar: 25 μm.

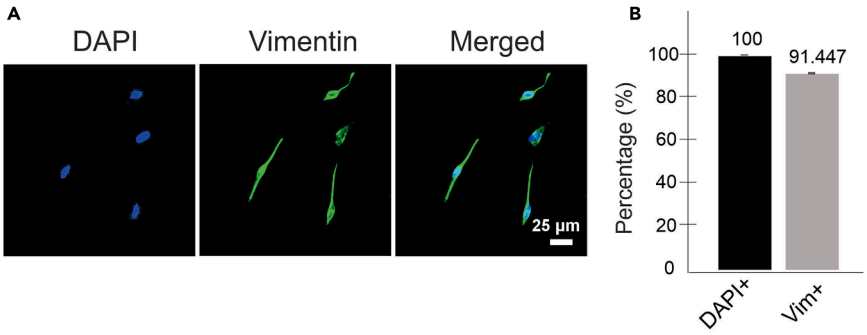

Figure 5. Fluorescence microscopy analysis and quantification of Müller glia cell purity in primary porcine Müller glia cultures

(A) Indirect fluorescence staining of primary porcine Müller glia cells for the Müller glia cell marker vimentin (green) counterstained with the nuclear DNA marker DAPI.

(B) Quantification of Müller glia cell purity in primary cultures of healthy porcine retina based on the number of vimentin-positive cells (Vim+) and their ratio to DAPI-positive nuclei (DAPI+). Analysis indicates that primary porcine Müller glial cell culture purity is greater than 90%, confirming the high specificity of the isolation procedure.

Cells isolated from 7-months-old wild-type porcine retina, passage number: 3. Counted cell number: 152, Scale bar: 25 μm.

TROUBLESHOOTING

Problem 1

Contamination of primary Müller glia cell cultures with RPE cells in the (Step 7).

Potential solution

RPE is located directly beneath the neural retina. RPE cells can be inadvertently removed during retinal dissection. Poor handling during retina isolation increases the contamination. As RPE cells proliferate rapidly in culture, they can quickly reach confluency, reducing both the yield and purity of Müller glia cultures. It is therefore necessary to take care to detach the retina very carefully from the underlying RPE layer using Dumont #3 and Dumont #4 forceps indicated in the protocol. Avoid scraping the RPE during removal. If contamination is observed, improve dissection technique and process tissue promptly to preserve retinal integrity.

Problem 2

Dislodging attached retinal pieces during the addition of Müller glia cell selection medium (Step 12c).

Potential solution

Add selection medium gently using serological or Pasteur pipettes, directing the flow to the bottom of the flask rather than onto the retinal pieces. When placing the flask vertically, move slowly and carefully to avoid detaching tissue. Adding medium too forcefully or directly onto the retinal tissue can wash away digested retinal pieces, which reduces the number of attached explants and lowering Müller glia cell yield. Rapid movements while positioning the flask vertically can also displace attached tissue.

Problem 3

Poor attachment of retinal pieces after mechanical digestion (Step 12d).

Potential solution

Insufficient contact between retinal pieces and the culture dish surface after mechanical digestion can limit attachment, leading to slower or less efficient Müller glia cell outgrowth. After mechanical digestion by using the 18 G angled needle, firmly press all retinal pieces onto the culture flask

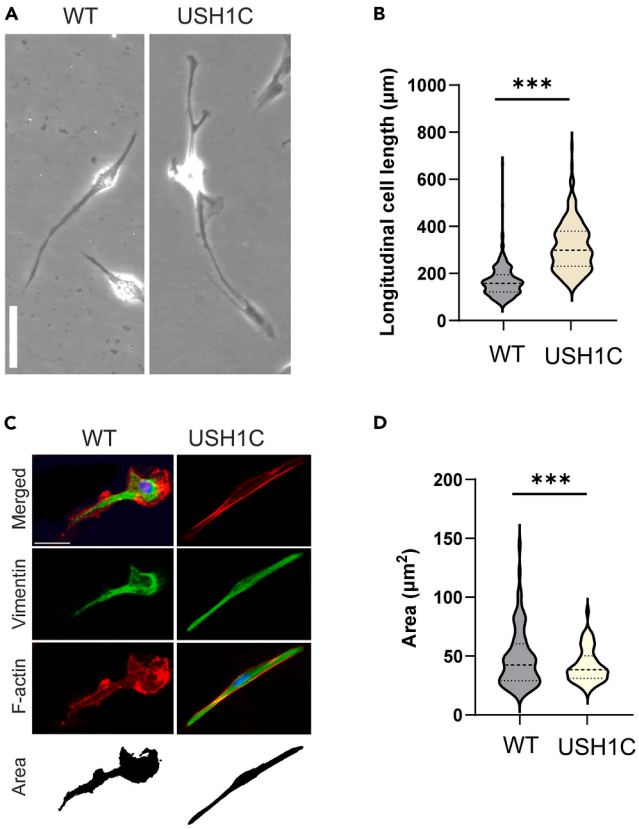

Figure 6. Phenotypic comparison of primary porcine Müller glia cells derived from WT and USH1C^{R31*} pigs

(A) Primary porcine Müller glia cells imaged by bright field microscope.

(B) Quantification of the length (measured by Fiji software) revealed that USH1C primary Müller glia cells are longer in comparison to healthy cells (WT).

(C) Fluorescence microscopy of primary Müller glia cells derived from USH1C and WT porcine retina. Cells were stained for the Müller glia cell marker vimentin by anti-vimentin (green), F-actin by TRITC-phalloidin (red), and by the nuclear DNA marker DAPI. F-actin staining allows to determine the area occupied by the cell shown as black areas (Step 78).

(D) Quantification of primary Müller glia cell areas (measured by Fiji) revealed that the area of USH1C primary Müller glia cells is smaller.

Quantifications by GraphPad Prism Software applying Kruskal-Wallis test, $p \leq 0.001$, ***; n:3. Scale bars: A: 100 µm, C: 25 µm.

All analysed primary Müller glia cells were isolated from 7-months-old wild-type or USH1C^{R31*} porcine retinae and imaged at passage number: 3.

surface and create a clear scratch with the needle tip. This improves tissue adhesion to the surface and enhances subsequent Müller glia cell outgrowth and yield.

Problem 4

Fungal and bacterial contamination in the primary Müller cell glia cultures (Steps 13 and 14).

Potential solution

Pigs are kept under non-sterile conditions, and despite initial sterilization of the eyeball with DPBS and iodine solution, contamination can occur during retinal isolation. This can lead to bacterial or fungal overgrowth early in culturing, compromising cell viability and purity. To minimize contamination risk, include penicillin-streptomycin (antibiotic) and amphotericin B (antimycotic) in the selection medium at the beginning of Müller cell glia isolation. Maintain strict aseptic techniques throughout all steps and process the tissue promptly after dissection.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Uwe Wolfrum (wolfrum@uni-mainz.de).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contacts, Yesim Tütüncü (ytuetuen@uni-mainz.de) and Uwe Wolfrum (wolfrum@uni-mainz.de).

Materials availability

Reagents and resources used in this study are commercially available. Nevertheless, requests for resources and reagents can be directed to the lead contact (wolfrum@uni-mainz.de), with a copy to the technical contact (ytuetuen@uni-mainz.de).

Data and code availability

This study did not generate new datasets.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.T. and U.W.; investigation, Y.T.; animal housing and breeding, N.K. and J.M.; writing, Y.T. and U.W.; review and editing, Y.T., N.K., J.M., and U.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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