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Title: Isolation and Culture of Primary Retinal Müller Cells from Sprague-Dawley (SD) Rats

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

   Scope shots: **2.5.1,2.6.1,2.6.2,2.6.3,2.6.4,2.7.1,2.7.2,2.7.3.**

   NOTE: The Videographer filmed the scope shots

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. Filming location: Will the filming need to take place in multiple locations?

Current Protocol Length

Number of Steps: 24
Number of Shots: 50 (8 Scope)

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Yunhua Tang:** This protocol describes isolation and culture of primary retinal müller cells from Sprague-Dawley (SD) Rats, which can aid in retinal research in the scientific community. The protocol covers eyeball enucleation, retinal dissection, cell extraction and identification, and key culture considerations.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.1*

What advantage does your protocol offer compared to other techniques?

1.2. **Yunhua Tang:** This protocol establishes an efficient, standardized and cost effective method for extracting and culturing RMCs from neonatal SD rats.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.1*

How will your findings advance research in your field?

1.3. **Yunhua Tang:** The in vitro RMC model can be used to simulate pathological conditions such as diabetic retinopathy and to assess drug effects.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.1.1*

Ethics Title Card

This research has been approved by the Animal Ethics Committee at the Chengdu University of Traditional Chinese Medicine

Protocol

2. Removal of the Eye from the Animal and Dissection of the Retina

Demonstrator: Yunhua Tang

2.1. To begin, pour D-Hank's solution into two 10-centimeter glass culture dishes **[1]**.

2.1.1. WIDE: Talent pouring D-Hank's solution into two separate 10 cm glass culture dishes placed on a clean bench.

2.2. After euthanizing and disinfecting the neonatal SD rat, position it on a sterile curved dish **[1-TXT]**. Using toothed tweezers, tear the eyelid skin along the palpebral fissure to expose the rat's eyeball **[2]**.

2.2.1. Talent placing the euthanized animal on the curved dish. **TXT: Euthanasia: Cervical dislocation (without CO_2 asphyxiation)**

2.2.2. Talent holding the animal and using toothed tweezers to gently tearing the eyelid skin along the palpebral fissure, revealing the eyeball.

2.3. Hold toothless tweezers open and parallel to the palpebral fissure to press down the orbital **[1]**. Once the optic nerve is reached and the eyeball is exposed, close the tweezers to lift and extract the eyeball **[2]**.

2.3.1. Talent positioning toothless tweezers and pressing down on the orbital with the tweezers held open.

2.3.2. Talent closing the tweezers and lifting the eyeball carefully once the optic nerve is reached.

2.4. Now, place the eyeball in a glass culture dish with D-Hank's solution **[1].** Rinse the eyeball **[2]** and transfer it to another dish with fresh D-Hank's solution **[3]**.

2.4.1. Talent placing the extracted eyeball into the first culture dish containing D-Hank's solution.

2.4.2. Talent gently shaking the eyeball in the solution.

2.4.3. Talent transferring the eyeball to a second culture dish with fresh D-Hank's solution.

2.5. Then, using curved ophthalmic micro forceps, gently fix the region between the cornea and optic nerve to expose the cornea **[1]**.

2.5.1. SCOPE: using curved ophthalmic micro forceps to hold the tissue between the cornea and optic nerve, exposing the cornea.

2.6. Pierce the corneoscleral junction using micro corneal scissors **[1]** and cut along the limbus in a circular fashion **[2]**. Make two symmetrical scleral incisions approximately 2 millimeters in length **[3]** before releasing the forceps and re-clamping at the junction of the optic nerve and sclera **[4]**.

2.6.1. SCOPE: piercing the corneoscleral junction using micro corneal scissors.

2.6.2. SCOPE: cutting along the limbus in a circular manner.

2.6.3. SCOPE: making two symmetrical 2 millimeter scleral incisions.

2.6.4. SCOPE: releasing the forceps and re-clamping them at the optic nerve and sclera junction.

2.7. Then, use a second forceps to gently press near the optic nerve root, directing pressure toward the corneal-optic nerve interface **[1]**. When the lens tissue appears, remove it carefully **[2]** and continue pressing until the retinal tissue emerges **[3]**.

2.7.1. SCOPE: using a second forceps to apply pressure near the optic nerve root.

2.7.2. SCOPE: carefully removing the lens tissue as it appears.

2.7.3. SCOPE: pressing the region until the retinal tissue is exposed.

2.8. Using forceps, transfer the separated retinal tissue to another sterile culture dish **[1]**.

2.8.1. Talent transferring the retinal tissue into a fresh sterile culture dish using forceps.

3. Extraction of Primary Retinal Müller cells (RMCs)

3.1. Open the culture dish lid **[1]** and use a pipette with a 1 milliliter tip to pipette the retinal tissue up and down about 15 times to break it into small pieces **[2]**.

3.1.1. Talent opening the lid of the culture dish.

3.1.2. Talent pipetting the retinal tissue up and down 15 times using a 1 milliliter pipette tip.

3.2. Then, incubate the tissue with 1 milliliter of 0.25 percent trypsin at 37 degrees Celsius for 5 minutes **[1]**.

3.2.1. Talent placing the culture dish into the incubator.

3.3. Remove the culture dish from the incubator and place it on the clean bench **[1]**. Add 2 milliliters of complete medium and pipette gently to stop the digestion **[2]**.

3.3.1. Talent removing the culture dish from the incubator and placing it on the clean bench.

3.3.2. Talent adding 2 milliliters of complete medium and gently pipetting the solution to stop trypsin digestion.

3.4. Now, filter the cell suspension through a 300-mesh nylon screen into a 15-milliliter centrifuge tube **[1]**. Wash the culture dish with prepared PBS **[2]** and collect the remaining suspension **[3]**.

3.4.1. Talent pouring the cell suspension on a 300-mesh nylon screen connected to a 15-milliliter centrifuge tube.

3.4.2. Talent adding the dish with phosphate-buffered saline.

3.4.3. Talent aspirating the rinse onto the filter.

3.5. Then, spin the tube at 878 *g* for 5 minutes at room temperature **[1]**. After centrifugation, aspirate and discard the supernatant **[2]**. Resuspend the pellet in 2 milliliters of complete medium **[3]** and centrifuge again at 878 *g* for 5 minutes to purify the cells **[4]**.

3.5.1. Talent placing the centrifuge tube in the centrifuge.

3.5.2. Talent aspirating and discarding the supernatant.

3.5.3. Talent resuspending the pellet in 2 milliliters of complete medium by pipetting up and down.

3.5.4. Talent placing the tube in a centrifuge.

3.6. After discarding the supernatant, resuspend the cells in 2 milliliters of complete medium **[1]**. Take T25 flask with 3 milliliters of complete medium and add 1 milliliter of the cell suspension **[2]**.

3.6.1. Talent resuspending the cells in 2 milliliters of complete medium by pipetting up and down.

3.6.2. Talent adding 3 milliliters of complete medium to a T25 flask, then pipetting 1 milliliter of cell suspension into the flask.

3.7. Shake the flask in a cross pattern before placing it in the incubator **[1]**.

3.7.1. Talent shaking the flask in a cross pattern.

3.8. After 48 hours of incubation, remove the flask from the incubator and place it on the clean bench **[1]**.

3.8.1. Talent removing the flask from the incubator.

3.9. Discard the spent medium **[1]** and wash the cell-adhering surface three times with 1 milliliter of PBS, which contains 1 percent penicillin plus streptomycin **[2]**.

3.9.1. Talent aspirating and discarding the used medium.

3.9.2. Talent adding the surface of the flask with 1 milliliter of PBS and shaking the flask.

3.10. Then, add 5 milliliters of fresh complete medium and continue the incubation until cell confluency exceeds 90% **[1-TXT]**.

3.10.1. Talent adding 5 milliliters of fresh complete medium to the flask. **TXT: Change the medium every other day**

4. Passaging of Retinal Müller cells (RMCs)

4.1. Wash the cells three times with 1 milliliter of PBS containing 1 percent penicillin-streptomycin **[1]**.

4.1.1. Talent adding 1 milliliter of PBS containing 1 percent penicillin+streptomycin to the cells and aspirating it.

4.2. Then, incubate the cells with 1 milliliter of 0.25 percent trypsin-EDTA solution for 1 minute and 30 seconds **[1]**.

4.2.1. Talent adding the cells with 1 milliliter of 0.25 percent trypsin-EDTA solution

4.3. Now, observe the flask under an inverted microscope **[1]**. When the cells appear round, detached, and begin to float, add 2 milliliters of complete culture medium to the flask

to terminate digestion **[2]**.

4.3.1. Talent placing the flask under an inverted microscope

~~4.3.2. SCOPE/Image: Show round, detached, and floating cells under the inverted microscope. **Authors,** please provide any correct image which may have been acquired earlier~~ **NOTE**: Omit this shot as authors did not respond, VO merged with the next shot

4.3.3. Talent adding 2 milliliters of complete culture medium and pipetting it up and down.

4.4. Then, use a pipette to aspirate the cell suspension and transfer the entire cell suspension to a 15-milliliter centrifuge tube **[2]**

4.4.1. Talent aspirating the cell suspension from the flask using a pipette and adding the aspirated cell suspension into a 15 milliliter centrifuge tube.

4.5. Rinse the flask wall with 2 milliliters of PBS containing 1 percent penicillin-streptomycin and add it to the same tube **[1]**. Centrifuge the tube at 878 *g* for 5 minutes at room temperature **[2]**.

4.5.1. Talent adding PBS to the flask and transferring the liquid to the tube.

4.5.2. Talent placing the centrifuge tube into the centrifuge.

4.6. Discard the supernatant **[1]** and resuspend the cell pellet in an appropriate volume of complete medium **[2]**. Finally, passage the cells at a ratio of 1 to 2 or 1 to 3 as required **[3-TXT]**.

4.6.1. Talent discarding the supernatant carefully using a pipette.

4.6.2. Talent resuspending the cell pellet in complete medium by pipetting up and down.

4.6.3. Talent adding the required volume of cell suspension into new flasks. **TXT: Assess the cells using microscopy and flow cytometry**

Results

5. Results

5.1. Second-passage retinal Müller cells or RMCs *(R-M-Cees)* displayed star-shaped or spindle-shaped morphologies, with round or oval nuclei and abundant cytoplasm **[1]**. Hematoxylin and eosin spindle- and star-shaped cells with abundant pink cytoplasm and centrally located oval nuclei, interconnected by fine filamentous structures **[2]**.
- 5.1.1. LAB MEDIA: Figure 1. *Video editor: sequentially highlight the 3 images*.
- 5.1.2. LAB MEDIA: Figure 2. *Video editor: sequentially highlight the 3 images*.

5.2. Immunofluorescence staining of staining of RMCs revealed strong red fluorescence in cells labeled for glutamine synthetase and aquaporin 4 **[1]**, and bright green fluorescence for CRALBP *(kral-B-P)*, Kir4.1 *(K-I-R-4-point-1)*, and vimentin **[2]**.
- 5.2.1. LAB MEDIA: Figure 3. *Video editor: Emphasize the images showing vivid red fluorescence labeled "GS, AQP4".*
- 5.2.2. LAB MEDIA: Figure 3. *Video editor: Highlight the images showing green fluorescence labeled CRALBP, Kir4.1, Vimentin*.

5.3. NeuN *(neu-N),* the negative control was not detected in immunofluorescence analysis, confirming the specificity of the RMC isolation **[1]**.
- 5.3.1. LAB MEDIA: Figure 3. *Video editor: Show the bottom row, focusing on the "NeuN" image*.

5.4. Flow cytometric analysis showed that 98.7% of the cells were positive for glutamine synthetase **[1]** and 97.0% were positive for CRALBP, indicating a high purity of the RMCs **[2]**.
- 5.4.1. LAB MEDIA: Figure 4. *Video editor: Highlight the rightward-shifted red peak labeled 98.7% in the GS histogram*.
- 5.4.2. LAB MEDIA: Figure 4. *Video editor: Highlight the red peak labeled 97.0% in the CRALBP histogram*.

Pronunciation guide

1. D-Hank's (short for Dulbecco's Hank's Balanced Salt Solution)

- **Pronunciation link**: https://www.howtopronounce.com/d-hank-s
- **IPA**: /di hæŋks/

- **Phonetic Spelling**: dee hanks

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2. Palpebral

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/palpebral
- **IPA**: /ˈpælprə-bəl/
- **Phonetic Spelling**: pal-pruh-buhl

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3. Optic

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/optic
- **IPA**: /ˈɑːptɪk/
- **Phonetic Spelling**: op-tik

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4. Corneoscleral

- **Pronunciation link**: https://www.howtopronounce.com/corneoscleral
- **IPA**: /ˌkɔrni.oʊˈsklɛrəl/
- **Phonetic Spelling**: kor-nee-oh-skler-uhl

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5. Limbus

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/limbus
- **IPA**: /ˈlɪmbəs/
- **Phonetic Spelling**: lim-buhs

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6. Scleral

- **Pronunciation link**: https://www.merriam-webster.com/medical/scleral
- **IPA**: /ˈsklɪrəl/
- **Phonetic Spelling**: sklair-uhl

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7. Retinal

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/retinal
- **IPA**: /ˈrɛtənəl/
- **Phonetic Spelling**: reh-tuh-nuhl

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8. Trypsin

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/trypsin
- **IPA**: /ˈtrɪpsɪn/
- **Phonetic Spelling**: trip-sin

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9. EDTA

- **Pronunciation link**: https://www.howtopronounce.com/edta
- **IPA**: /ˌiːdiːtiːˈeɪ/
- **Phonetic Spelling**: ee-dee-tee-ay

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10. Vimentin

- **Pronunciation link**: https://www.howtopronounce.com/vimentin
- **IPA**: /ˈvɪməntɪn/
- **Phonetic Spelling**: vih-men-tin

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11. CRALBP

- **Pronunciation link**: https://www.howtopronounce.com/cralbp
- **IPA**: /kræl bi pi/
- **Phonetic Spelling**: kral-bee-pee

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12. Kir4.1

- **Pronunciation link**: https://www.howtopronounce.com/kir4-1
- **IPA**: /kɪr fər pɔɪnt wʌn/

- **Phonetic Spelling**: kir four point one

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13. Aquaporin

- **Pronunciation link**: https://www.merriam-webster.com/medical/aquaporin
- **IPA**: /ˌɑːkwəˈpɔːrɪn/
- **Phonetic Spelling**: ah-kwuh-por-in

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14. Glutamine

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/glutamine
- **IPA**: /ˈgluːtəˌmiːn/
- **Phonetic Spelling**: gloo-tuh-meen

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15. Müller

- **Pronunciation link**: https://www.howtopronounce.com/müller-cells
- **IPA**: /ˈmʊlər/
- **Phonetic Spelling**: moo-ler

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16. NeuN

- **Pronunciation link**: https://www.howtopronounce.com/neun
- **IPA**: /nuː ɛn/
- **Phonetic Spelling**: noo-en
