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Title: Isolation of Pericytes from Mouse Cortical Tissue Using FACS for Single-Cell Sequencing

Authors and Affiliations:

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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, all done

- 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? **NO**

- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 30

Number of Shots: 58

Interviews

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

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Xinyi Cui:** Our research examines brain pericytes, focusing on their vascular roles and interactions within the neurovascular unit to understand cerebral function.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~What technologies are currently used to advance research in your field?~~

- 1.2. **Xinyi Cui:** Live imaging and spatial omics enable precise mapping of pericyte function within brain vasculature, revealing location-specific roles and therapeutic targets.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.5. **Qingbin Wu:** This protocol allows researchers to isolate high-purity, viable pericytes suitable for downstream single-cell sequencing and functional analyses.
 - 1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.6. **Xinyi Cui:** The main challenge is the absence of highly specific pericyte markers, complicating pure isolation and increasing the risk of cellular contamination.
 - 1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

1.7. **Qingbin Wu:** Future studies can focus on identifying pericyte-specific markers, enabling improved isolation, deeper functional characterization, and targeted therapeutic investigations.

1.7.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

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

Ethics Title Card

This research has been approved by the Laboratory Animal Care and Ethics Committee of the Institute of Microcirculation, Chinese Academy of Medical Sciences, and Peking Union Medical College

Protocol

NOTE: SCOPE timestamps for protocol were added at the postshoot stage. Please contact the postshoot note integrator (Sri Lakshmi Priya) for queries regarding lab media.

2. Mouse Brain Dissection and Cerebral Cortex Isolation

Demonstrator: Xinyi Cui

- 2.1. To begin, spray the head and neck region of an anesthetized mouse with 75 percent ethanol [1-TXT]. Using a scalpel, make a midline scalp incision from the occipital to the frontal region to expose the skull [2].
 - 2.1.1. WIDE: Talent spraying the head and neck region with ethanol. **TXT: Anesthesia: Tribromoethanol (0.2 mL/10 g body weight) (i.p)**
 - 2.1.2. Talent making a midline scalp incision to expose the skull.
- 2.2. Perform a transverse incision anterior to the bilateral orbits [1], followed by lateral incisions along the inferior margins of the cerebellum to detach the skull base [2].
 - 2.2.1. Talent performing a transverse incision anterior to both orbits.
 - 2.2.2. Talent making lateral incisions along the inferior margins of the cerebellum.
- 2.3. Incise the calvarium longitudinally along the sagittal suture from posterior to anterior [1]. Lift the cranial flap together with the dura mater to expose the brain [2].
 - 2.3.1. Talent cutting along the sagittal suture of the skull.
 - 2.3.2. Talent lifting the cranial flap to reveal the brain.
- 2.4. Using surgical scissors, gently elevate the brain while severing the optic nerves and the medulla oblongata–spinal cord connection [1].
 - 2.4.1. Talent cutting the optic nerves and brainstem connection with scissors.
- 2.5. Place the intact brain gently into a 10-centimeter dish containing 10 milliliters of ice-cold PBS (*P-B-S*), avoiding any damage [1]. ~~Handle the tissue gently to avoid damage [2].~~ NOTE: For removed shot, VO is added in concise format in previous step
 - 2.5.1. Talent placing the intact brain into a dish with cold buffer.
 - 2.5.2. ~~Talent handling the tissue gently.~~ NOTE: Author removed the shot and added the VO concisely with previous step.
- 2.6. Transfer the brain onto aluminum foil [1]. ~~Using forceps, lift the dura mater from the margins and excise it along the midline sulci [2]. After complete dura removal, expose the subarachnoid space [3].~~
 - 2.6.1. Talent placing the brain on aluminum foil.

2.6.2. SCOPE: Talent removing the dura mater with forceps. Author's NOTE: Remove it

2.6.3. SCOPE: JOVE_LM_69749_Step2.6.2_Shot-showing-the-exposed-subarachnoid-space..mp4: 00:07-00:19

2.7. Rinse the subarachnoid space with Hanks balanced salt solution [1]. Transfer the tissue to filter paper and remove the pia mater by rolling [2].

2.7.1. SCOPE:JOVE_LM_69749_Step2.7.1_Talent-pipetting-HBSS-buffer-over-the-subarachnoid-space..mp4: 00:00-00:27

2.7.2. SCOPE:JOVE_LM_69749_Step2.7.2_Talent-transferring-the-tissue-to-filter-paper-and-rolling-it-to-remove-the-pia-mater..mp4: 00:03-00:32

2.8. With a razor blade, cut and discard the olfactory bulb and the caudal-most sections [1] and section the brain coronally into slices 2 to 3 millimeters thick [2]. NOTE: VO is also edited for inverted shots.

2.8.2. SCOPE:JOVE_LM_69749_Step2.8.1_Talent-cutting-the-brain-into-coronal-sections-and-discarding-unwanted-sections..mp4: 00:00-01:24

2.8.1 SCOPE:JOVE_LM_69749_Step2.8.1_Talent-cutting-the-brain-into-coronal-sections-and-discarding-unwanted-sections..mp4: 00:00-01:24

NOTE: 2.8.2 was placed before 2.8.1 as per the scope video. Show the scope shot only once.

Author's NOTE: 2.8.1 and 2.8.2 are combined in one shot.

2.9. Isolate the cerebral cortex along the corpus callosum [1]. Transfer the cortical tissue into ampoules containing 3 milliliters of ice-cold tissue collection buffer [2].

2.9.1. SCOPE: JOVE_LM_69749_Step2.9.1_Talent-isolating-the-cerebral-cortex..mp4: 00:13-00:42, 01:18-01:52

2.9.2. Talent transferring cortical tissue into ampoules with buffer.

3. Tissue Dissociation and Density Gradient–Based Cell Enrichment

3.1. Using surgical scalpels, mince the tissue in ampoules into fragments of approximately 1 by 1 millimeter and complete the mincing within 5 minutes to preserve tissue freshness [1].

3.1.1. Talent mincing tissue with scalpels.

3.2. Pre-rinse pipette tips with tissue collection buffer [1]. Then slowly aspirate the minced fragments toward the tip apex while holding the pipette vertically [2]. Gently expel the fragments into pre-chilled 15-milliliter centrifuge tubes [3].

3.2.1. Talent rinsing pipette tips with buffer.

- 3.2.2. Talent aspirating tissue fragments vertically.
- 3.2.3. Talent dispensing fragments into centrifuge tubes.
- 3.3. Wash the original ampoules with 2 percent FBS (*F-B-S*) in PBS saline [1]. Transfer the wash into the same centrifuge tubes and centrifuge at 300 g for 5 minutes at 4 degrees Celsius [2].
 - 3.3.1. Talent rinsing ampoules with 2% FBS in PBS.
 - 3.3.2. Talent transferring the wash into centrifuge tubes.
- 3.4. Discard the supernatant after centrifugation [1]. Resuspend each pellet in 3 milliliters of CD (*C-D*) enzyme working solution [2]. Aliquot the suspension equally into three tubes and add additional enzyme solution to reach a final volume of 5 milliliters per tube [3].
 - 3.4.1. Talent aspirating and discarding supernatants.
 - 3.4.2. Talent resuspending pellets in enzyme solution.
 - 3.4.3. Talent aliquoting the suspension into 3 tubes and adjusting final volume to 5 ml with enzyme solution.
- 3.5. Incubate the tubes in a hybridization oven at 37 degrees Celsius for 100 minutes with orbital shaking at 100 g [1].
 - 3.5.1. Talent placing tubes into the oven.
- 3.6. During incubation, add 1 milliliter of DNase I (*D-N-Ase-One*) solution to 10 milliliters of 2 percent FBS in PBS [1]. Terminate digestion centrifuging at 300 g at 4 degrees Celsius for 5 minutes [2].
 - 3.6.1. Talent preparing the dissociation solution at the bench.
 - 3.6.2. Talent loading tubes into the centrifuge.
- 3.7. Then resuspend each pellet in 1 milliliter of tissue dissociation solution [1]. Pipette the solution 100 times to aid mechanical dissociation [2].
 - 3.7.1. Talent resuspending the pellet in 1 mL of tissue dissociation solution.
 - 3.7.2. Talent repeatedly pipetting to dissociate tissue.
- 3.8. Next, pool all suspensions into a single 50-milliliter centrifuge tube [1]. Rinse each tube with 2 milliliters of buffer and combine the washes [2].
 - 3.8.1. Talent pooling cell suspensions.
 - 3.8.2. Talent rinsing and combining washes.
- 3.9. After centrifugation at 300g for 5 minutes at 4 degrees Celsius and discarding the uppermost layer, add 12.5 milliliters of 20 percent BSA (*B-S-A*) and invert to mix [1]. Centrifuge at 1,000 g at 4 degrees Celsius for 20 minutes [2].

- 3.9.1. Talent adding bovine serum albumin solution and inverting the tubes
- 3.9.2. Talent placing the tube into the centrifuge.
- 3.10. Now collect the bottom pellet and transfer the supernatant into a new 50 milliliter centrifuge tube [1]. Resuspend gently before centrifuging again [2].
 - 3.10.1. Talent separating pellet and supernatant.
 - 3.10.2. Talent loading tubes for the second centrifugation.
- 3.11. Resuspend each pellet in 2 milliliters of 2 percent FBS in PBS [1]. Then combine both suspensions into a single tube [2].
 - 3.11.1. Talent resuspending pellets in 2 mL of FBS in PBS.
 - 3.11.2. Talent combining the suspensions.
- 3.12. Centrifuge the combined suspension at 300 g at 4 degrees Celsius for 5 minutes [1]. After discarding the supernatant, resuspend the final pellet in 2 milliliters of PBS with 2% FBS [2].
 - 3.12.1. Talent placing the combined suspension in a centrifuge.
 - 3.12.2. Talent resuspending the final purified cells in 2 mL PBS.
- 3.13. Prepare four 15-milliliter centrifuge tubes containing 4 milliliters of 22 percent Percoll solution [1]. Gently layer 0.5 milliliter aliquots of the cell suspension onto each gradient and centrifuge at 560g for 10 minutes at 4 degrees Celsius [2].
 - 3.13.1. Talent preparing Percoll gradients.
 - 3.13.2. Talent layering cell suspensions onto the gradients.
- 3.14. After aspirating the supernatant, resuspend each pellet in 1 milliliter of HBSS (H-B-S-S) BSA glucose buffer [1]. Then pool the suspensions into a new 15-milliliter tube [2]. Rinse the Percoll tubes with 2 milliliters of buffer and combine the washes before centrifugation at 300 g for 5 minutes at 4 degrees Celsius [3].
 - 3.14.1. Talent resuspending Percoll pellets in 1 mL HBSS/BSA/Glucose buffer.
 - 3.14.2. Shot of the suspensions being pooled into a 15 mL tube.
 - 3.14.3. Shot of the washes being combined into the 15 mL tube.

4. Flow Cytometry Staining and Preparation of Brain-Derived Cells for Sorting

- 4.1. For flow cytometry, resuspend the pellet in 1 milliliter of HBSS BSA glucose buffer after aspirating the supernatant [1].
 - 4.1.1. Talent resuspending the cell pellet in 1 mL of HBSS/BSA/glucose buffer.

- 4.2. Add 5 microliters of Fc (F-C) receptor blocking reagent and incubate at 4 degrees Celsius for 5 minutes [1]. Then, pipette fluorescently conjugated antibodies to the suspension before incubating [2].

4.2.1. Talent adding Fc receptor blocking reagent and placing the tube on ice.

4.2.2. Talent adding antibody reagents to the tube.

AND

TEXT ON PLAIN BACKGROUND:

Fluorescently conjugated antibodies:

5 µL of CD45-PE

5 µL of CD41-PE

20 µL of CD31-APC

15 µL of CD13-FITC

Incubation: On ice, 20 min in the dark

Video Editor: Please play both shots side by side in a split screen

- 4.3. Wash the cells with 6 milliliters of HBSS BSA glucose buffer [1]. Then centrifuge the suspension at 300 g at 4 degrees Celsius for 5 minutes [2].

4.3.1. Talent adding 6 mL wash buffer to the cells

4.3.2. Talent placing the stained cells in a centrifuge.

- 4.4. After aspirating the supernatant, resuspend the pellet in 1 milliliter of buffer [1]. Add 200 microliters of 7-aminoactinomycin D solution [2] and incubate on ice for 10 minutes [3]. Then, adjust the final volume to 2 milliliters with HBSS buffer supplemented with BSA and glucose [4]. NOTE: VO is added for additional shot

4.4.1. Talent resuspending the pellet in 1mL buffer.

4.4.2. Talent adding 200 µL of AAD solution.

4.4.3. Talent incubating the tube on ice.

Added shot: Talent adding buffer to bring the final volume to 2 mL

- 4.5. Rinse the inner surface of sorting collection tubes with 1 percent BSA solution [1]. Aspirate out the excess solution, leaving exactly 1 milliliter of solution in each tube [2]. Store the tubes on ice to receive sorted cells [3]. NOTE: VO has been edited to match the revised shot.

4.5.1. ~~Talent coating sorting tubes with bovine serum albumin solution.~~ Talent swirling the sorting collection tubes containing 1% BSA solution

4.5.2. Shot of the excess solution being pipetted out.

4.5.3. Shot of prepared collection tubes resting on ice.

Results

5. Results

- 5.1. Cells dissociated from brain tissue were sequentially gated by forward scatter area and side scatter area to exclude cellular debris during flow cytometry sorting [1]. CD45 (*C-D-Forty-five*) -positive and CD41(*C-D-Forty-one*) -positive dual-positive events were excluded to remove hematopoietic cells and platelet contaminants [2].
 - 5.1.1. LAB MEDIA: Figure 2. *Video editor: Highlight the top-left plot (SSC-A) and focus on the boxed region*
 - 5.1.2. LAB MEDIA: Figure 2. *Video editor: Highlight the top-right plot.*
- 5.2. CD13-positive vascular wall cells were enriched, and apoptotic cells were excluded using 7-aminoactinomycin D negative gating, followed by CD31-negative selection to isolate pericytes [1].
 - 5.2.1. LAB MEDIA: Figure 2. *Video editor: Highlight the gated region in the bottom-left plot.*
- 5.3. Flow cytometry sorting showed that nucleated cells accounted for 59.8 percent of all events [1], CD13-positive cells accounted for 1 percent of all events [2], and the final CD13-positive CD31-negative pericyte population accounted for 0.7 percent of all events [3].
 - 5.3.1. LAB MEDIA: Figure 2. *Video editor: Highlight the table entry showing 59.8 percent for nucleated cells.*
 - 5.3.2. LAB MEDIA: Figure 2. *Video editor: Highlight the table entry showing 1.0 percent for the CD13-positive population.*
 - 5.3.3. LAB MEDIA: Figure 2. *Video editor: Highlight the table entry showing 0.7 percent for the CD13-positive CD31-negative population.*
- 5.4. UMAP (*You-Map*) dimensionality reduction of the sorted cells revealed six distinct major clusters, indicating cellular heterogeneity within the isolated population [1].
 - 5.4.1. LAB MEDIA: Figure 3A. *Video editor: Show the full UMAP plot with the six color-coded clusters.*
- 5.5. Z-score normalized heatmap analysis demonstrated that pericyte marker genes were highly and predominantly expressed in clusters 0 through 3 [1].
 - 5.5.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the red boxes in the rows corresponding to clusters 0 to 3*

5.6. Cluster proportion analysis showed that clusters 0 through 3 constituted 87.1 percent of the total population [1], whereas smooth muscle cell markers were enriched in cluster 4 at 10.14 percent [2] and fibroblast markers were enriched in cluster 5 at 2.77 percent [3].

5.6.1. LAB MEDIA: Figure 3C. *Video editor: please highlight the bars corresponding to Clusters 0 to 3*

5.6.2. LAB MEDIA: Figure 3C. *Video editor: please highlight the bars corresponding to Clusters 4*

5.6.3. LAB MEDIA: Figure 3C. *Video editor: please highlight the bars corresponding to Clusters 5*

Pronunciation guides:

1. Anesthetized
Pronunciation link: <https://www.merriam-webster.com/dictionary/anesthetized>
IPA: /əˈnesθəˌtaɪzd/
Phonetic Spelling: uh·ness·thuh·tyzd
2. Occipital
Pronunciation link: <https://www.merriam-webster.com/dictionary/occipital>
IPA: /ɑkˈsɪpətəl/
Phonetic Spelling: ok·sip·uh·tuhl
3. Ethanol
Pronunciation link: <https://www.merriam-webster.com/dictionary/ethanol>
IPA: /ˈeθəˌnɒl/
Phonetic Spelling: eth·uh·nawl
4. Scalpel
Pronunciation link: <https://www.merriam-webster.com/dictionary/scalpel>
IPA: /ˈskalpəl/
Phonetic Spelling: skal·puhl
5. Cerebellum
Pronunciation link: <https://www.merriam-webster.com/dictionary/cerebellum>
IPA: /ˌserəˈbeləm/
Phonetic Spelling: seh·ruh·bel·uhm
6. Calvarium
Pronunciation link: <https://www.merriam-webster.com/dictionary/calvarium>
IPA: /kalˈveriəm/
Phonetic Spelling: kal·vair·ee·uhm

7. Sagittal
Pronunciation link: <https://www.merriam-webster.com/dictionary/sagittal>
IPA: /'sædʒɪtəl/
Phonetic Spelling: saj·uh·tuhl
8. Dura Mater
Pronunciation link: <https://www.merriam-webster.com/dictionary/dura%20mater>
IPA: /,dʊrə 'mætər/
Phonetic Spelling: dur·uh maa·ter
9. Optic
Pronunciation link: <https://www.merriam-webster.com/dictionary/optic>
IPA: /'ɒptɪk/
Phonetic Spelling: op·tik
10. Medulla Oblongata
Pronunciation link: <https://www.merriam-webster.com/dictionary/medulla%20oblongata>
IPA: /məˌdʌlə ˌɒblŋɡətə/
Phonetic Spelling: muh·dul·uh ob·long·gaa·tuh
11. Subarachnoid
Pronunciation link: <https://www.merriam-webster.com/dictionary/subarachnoid>
IPA: /,sʌbə'rækˌnɔɪd/
Phonetic Spelling: sub·uh·rak·noyd
12. Hanks
Pronunciation link: <https://www.merriam-webster.com/dictionary/Hanks>
IPA: /'hæŋks/
Phonetic Spelling: hanks
13. Pia Mater
Pronunciation link: <https://www.merriam-webster.com/dictionary/pia%20mater>
IPA: /,paɪə 'mætər/
Phonetic Spelling: pie·uh maa·ter
14. Olfactory
Pronunciation link: <https://www.merriam-webster.com/dictionary/olfactory>
IPA: /ɒl'fæktəri/, /oʊl'fæktəri/
Phonetic Spelling: ol·fak·tor·ee
15. Coronally
Pronunciation link: <https://www.merriam-webster.com/dictionary/coronal>
IPA: /kə'roʊnəl/
Phonetic Spelling: kuh·roh·nuhl·ee
16. Cerebral Cortex
Pronunciation link: <https://www.merriam-webster.com/dictionary/cerebral%20cortex>
IPA: /sə'ri:brəl 'kɔrtɛks/
Phonetic Spelling: suh·ree·bruhl kor·teks
17. Corpus Callosum
Pronunciation link: <https://www.merriam-webster.com/dictionary/corpus%20callosum>

IPA: /ˌkɔrpəs kəˈloʊsəm/

Phonetic Spelling: kor·pus kuh·loh·suhm

18. Ampoules

Pronunciation link: <https://www.merriam-webster.com/dictionary/ampoule>

IPA: /ˈæmˌpuːl/

Phonetic Spelling: am·pool

19. Dissociation

Pronunciation link: <https://www.merriam-webster.com/dictionary/dissociation>

IPA: /dɪˌsoʊsiˈeɪʃən/

Phonetic Spelling: dih·soh·see·ay·shuhn

20. Aliquot

Pronunciation link: <https://www.merriam-webster.com/dictionary/aliquot>

IPA: /ˈælɪˌkwɒt/

Phonetic Spelling: al·ih·kwot

21. Centrifuge

Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>

IPA: /ˈsentərəˌfjuːdʒ/

Phonetic Spelling: sen·truh·fyooj

22. Supernatant

Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>

IPA: /ˌsuːpərˈnextənt/

Phonetic Spelling: soo·per·nay·tuhnt

23. Resuspend

Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspend>

IPA: /ˌriːsəˈspend/

Phonetic Spelling: ree·suh·spend

24. Pellet

Pronunciation link: <https://www.merriam-webster.com/dictionary/pellet>

IPA: /ˈpelət/

Phonetic Spelling: pel·uht

25. Enzyme

Pronunciation link: <https://www.merriam-webster.com/dictionary/enzyme>

IPA: /ˈenzaim/

Phonetic Spelling: en·zym

26. Tribromoethanol

Pronunciation link: No confirmed link found

IPA: /traɪˌbroʊmoʊˈeθəˌnɒl/

Phonetic Spelling: try·broh·moh·eth·uh·nawl

27. Coronal

Pronunciation link: <https://www.merriam-webster.com/dictionary/coronal>

IPA: /kəˈroʊnəl/

Phonetic Spelling: kuh·roh·nuhl