FINAL SCRIPT: APPROVED FOR FILMING

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Title: Flow Cytometry and Single-Cell Analysis for Characterizing Microglia Activation in Early Postnatal Mouse Brain Development

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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? **No**

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? **Yes**

   The centrifuge and the water bath are located in different rooms.

Current Protocol Length
Number of Steps: 24
Number of Shots: 54

Introduction

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

1.1. **Marianne Mengus:** Our research explores how microglia influence brain repair after perinatal cerebellar injury. We aim to determine whether modulating microglial cell activity can improve long-term neurological outcomes **[1].**

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

What are the most recent developments in your field of research?

1.2. **Sophie Tremblay:** I believe the complexity of microglial cell responses and the challenges of describing those most accurately to represent physiological, but also pathological responses in diseases **[1].**

1.2.1. 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?

1.3. **Marianne Mengus:** We are using different modalities, such as single-cell RNA sequencing and flow cytometry experiments, to characterize microglial states and functions in the developing brain after perinatal injury **[1].**

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 6*

What are the current experimental challenges?

1.4. **Marianne Mengus:** A major challenge is preserving cell viability and microglia identity during isolation, especially at neonatal time points when cerebellar cell numbers are limited **[1].**

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

What new scientific questions have your results paved the way for?

1.5. **Benjamin Boucher:** This raises important questions about how early-life cerebellar insults lead to transcriptomic alterations in specific microglial subpopulations, and how targeted therapeutic interventions might modulate these changes **[1].**

1.5.1. 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 ethical committee of the CHU Sainte-Justine Research Centre and complies with the guidelines and policies of the Ste-Justine Research Center and the University of Montreal

Protocol

2. Cell Isolation from the Mice's Brain

Demonstrator: Marianne Mengus

2.1. To begin, dissect the specific brain region of mice as needed **[1]**. Using forceps, transfer the tissue into a small Petri dish containing microglial cell culture medium placed on ice to maintain cell viability **[2]**.

2.1.1. WIDE: Talent with the dissected brain in front of him.

2.1.2. Talent placing dissected tissue into a small Petri dish filled with cold microglial cell culture medium.

2.2. Using a pipette, remove the microglial cell culture medium from the Petri dish **[1]**. Then, using a scalpel, finely cut the brain tissue in the same Petri dish **[2]**.

2.2.1. Talent removing the microglial medium from the dish with a pipette.

2.2.2. Talent slicing the brain tissue finely using a scalpel in the Petri dish.

2.3. Transfer the homogenized brain tissue into 5-milliliter tubes for enzymatic digestion **[1]**. Add 2 milliliters of Hank's Balanced Salt Solution supplemented with collagenase D and Dnase I to each tube **[2-TXT]** and seal the caps tightly with parafilm **[3]**. Then, incubate the tubes in a 37-degree Celsius water bath for 15 minutes, shaking them every 5 minutes **[4]**. To stop the digestion, place the tubes on ice **[5]**.

2.3.1. Talent transferring homogenized tissue into 5 milliliter tubes.

2.3.2. Talent pipetting enzyme mix into the tubes. **TXT: Collagenase D: 2 mg/mL; DNase I: 14 µg/mL**

2.3.3. Talent sealing the tubes with parafilm.

2.3.4. Talent placing tubes in a 37 degrees Celsius water bath and shaking them.

2.3.5. Talent transferring tubes onto ice.

2.4. Then, pass the homogenate through a 140-micrometer metal mesh filter to remove large debris **[1]**. Using a glass pestle, gently dissociate the remaining cells on the filter **[2]**.

2.4.1. Talent pouring the homogenate through a 140 micrometer metal mesh filter.

2.4.2. Talent using a glass pestle to break up residual cell clumps.

2.5. Wash the metal mesh filter multiple times with 3 milliliters of microglial cell culture medium for each wash **[1]**.

2.5.1. Talent pipetting 3 milliliters of microglial cell culture medium over the filter repeatedly.

2.6. Now, using a 10-milliliter pipette, collect the filtrate and transfer it into a 15-milliliter tube **[1]**. Then, centrifuge the tube at 500 *g* for 7 minutes at 4 degrees Celsius **[2]**.

2.6.1. Talent transferring the filtrate into a 15-milliliter tube using a 10-milliliter pipette.

2.6.2. Talent placing the tube in a centrifuge and setting it to 500 g for 7 minutes at 4 degrees Celsius.

2.7. Afterward, carefully invert the tube to discard the supernatant **[1]**. Using a rack, gently scrape the tube to resuspend the pellet **[2]**.

2.7.1. Talent inverting the centrifuge tube over a waste container to discard the supernatant.

2.7.2. Talent gently scraping the bottom of the tube on a rack to resuspend the pellet.

2.8. Then, add 10 milliliters of a 37 percent silica-based colloidal medium solution to the resuspended cells **[1]**. Centrifuge the solution at 500 *g* for 10 minutes at 4 degrees Celsius with minimal brake force **[2]**.

2.8.1. Talent pipetting 10 milliliters of silica-based colloidal solution into the tube.

2.8.2. Talent placing the tube in the centrifuge and setting it to 500 g for 10 minutes at 4 degrees Celsius with minimal braking.

2.9. Using a 10-milliliter pipette, aspirate the myelin layer from the top of the solution **[1]**.

2.9.1. Talent aspirating the upper myelin layer from the centrifuged tube using a 10-milliliter pipette.

2.10. To wash the cells, add 10 milliliters of Hank's Balanced Salt Solution **[1]** and centrifuge the tube at 500 *g* for 7 minutes at 4 degrees Celsius **[2]**.

2.10.1. Talent adding 10 milliliters of Hank's Balanced Salt Solution to the tube.

2.10.2. Talent centrifuging the tube at 500 g for 7 minutes at 4 degrees Celsius.

2.11. After discarding the supernatant and resuspending the pellet, add 10 milliliters of fluorescent-activated cell sorting buffer to the tube **[1]**. After centrifugation, resuspend the final pellet in the remaining buffer for downstream applications **[2-TXT]**. NOTE: VO is lightly modified for the second shot.

2.11.1. Talent adding 10 milliliters of fluorescent-activated cell sorting buffer.

2.11.2. Talent gently removes the supernatant by inverting the tube and showing the retained cells ready for further processing. **TXT: Centrifugation: 500 × *g*; 4 °C; 10 min** NOTE: This text overlay is moved from the first shot to the second shot to match the narration

3. Flow Cytometry Extracellular Staining Protocol

3.1. Transfer all isolated cells into a 96-well plate with a conical bottom **[1]**. Centrifuge the plate at 500 *g* for 5 minutes at 4 degrees Celsius **[2]**.

3.1.1. Talent pipetting cells into a 96-well conical-bottom plate.

3.1.2. Talent placing the plate in a centrifuge and running it at 500 g for 5 minutes at 4 degrees Celsius.

3.2. Quickly invert the plate in a single motion to discard the supernatant **[1]**. Then, resuspend the cell pellet in 25 microliters of blocking solution and incubate the plate at room temperature for 15 minutes **[2]**.

3.2.1. Talent inverting the 96-well plate over a waste container in one swift motion.

3.2.2. Talent pipetting 25 microliters of blocking solution into each well and mixing gently.

3.3. To prepare the extracellular antibody staining mix, centrifuge the antibody stock tubes at 10,000 *g* to remove aggregates **[1]**. Without disturbing the pellet, aspirate the required volume from the supernatant **[2]**. Using fluorescent-activated cell sorting buffer, adjust the total volume to 25 microliters **[3]**.

3.3.1. Talent placing antibody tubes in a high-speed centrifuge and running at 10,000 g.

3.3.2. Talent carefully aspirating supernatant from the centrifuged antibody tubes.

3.3.3. Talent adding fluorescent-activated cell sorting buffer to adjust the antibody mix to 25 microliters.

3.4. Now, add 25 microliters of the extracellular antibody staining mix to each well and incubate the plate for 20 minutes at room temperature **[1]**. Without mixing, add 150 microliters of fluorescent-activated cell sorting buffer to each well **[2]**. Centrifuge the plate at 500 *g* for 5 minutes at 4 degrees Celsius **[3]**. Then, invert the plate quickly to remove the supernatant in a single motion **[4]**.

3.4.1. Talent pipetting 25 microliters of antibody mix into each well.

3.4.2. Talent pipetting 150 microliters of buffer into each well without disturbing the contents.

3.4.3. Talent centrifuging the plate at 500 g for 5 minutes at 4 degrees Celsius.

3.4.4. Talent inverting the plate to discard the supernatant swiftly.

3.5. Resuspend the cell pellet in 200 microliters of fluorescent-activated cell sorting buffer and centrifuge as demonstrated earlier **[1-TXT]**.

3.5.1. Talent resuspending the pellet with 200 microliters of buffer. **TXT: Wash the cell pellet 2x**

4. Intracellular Staining

4.1. Resuspend the cells in 100 microliters of saponin-paraformaldehyde buffer to fix and permeabilize the cells **[1]**. Then, incubate the plate for 10 minutes at room temperature, protected from light **[2]**.

4.1.1. Talent pipetting 100 microliters of saponin-paraformaldehyde buffer into each well to resuspend the cells.

4.1.2. Talent covering the plate with foil and keeping it aside.

4.2. Without mixing, add 100 microliters of saponin buffer to each well **[1]**. Centrifuge the plate at 500 *g* for 6 minutes at 4 degrees Celsius **[2]**. Then, invert the plate to remove the supernatant in a single motion **[3]** and resuspend the cell pellet in 200 microliters of saponin buffer **[4]**.

4.2.1. Talent gently pipetting 100 microliters of saponin buffer into the wells without disturbing the contents.

4.2.2. Talent centrifuging the plate at 500 g for 6 minutes at 4 degrees Celsius.

4.2.3. Talent inverting the plate to discard the supernatant in one motion.

4.2.4. Talent resuspending the cell pellet in 200 microliters of saponin buffer.

4.3. Next, prepare compensation controls by adding 20 microliters of beads to 11 empty wells **[1-TXT]**.

4.3.1. Talent pipetting 20 microliters of beads into 11 designated wells for compensation controls. **TXT: Repeat centrifugation and discard the supernatant**

4.4. To prepare the intracellular antibody staining mix, centrifuge antibody stock tubes at 10,000 *g* to remove potential aggregates **[1]**. Without disturbing the pellet, aspirate the required volume from the supernatant **[2]**. Adjust the volume to 50 microliters using saponin buffer **[3]**.

4.4.1. Talent centrifuging antibody tubes at 10,000 g in a high-speed centrifuge.

4.4.2. Talent carefully aspirating from the supernatant of each tube without disturbing the pellet.

4.4.3. Talent adding saponin buffer to the aspirated antibodies to bring the volume to 50 microliters.

4.5. Then, resuspend the cell pellet in 50 microliters of intracellular antibody mix **[1]**. Add 1 microliter of each antibody to the respective bead wells and incubate the plate for 30 minutes at room temperature **[2]**.

4.5.1. Talent resuspending the pellet with 50 microliters of intracellular antibody mix.

4.5.2. Talent pipetting 1 microliter of each antibody into the designated bead wells and keeping it aside.

4.6. Without mixing, add 150 microliters of saponin buffer to each well-containing cell samples **[1]**. Add 100 microliters of fluorescent-activated cell sorting buffer to each compensation control well to wash the beads **[2]**.

4.6.1. Talent pipetting 150 microliters of saponin buffer into the cell wells without disturbing the contents.

4.6.2. Talent adding 100 microliters of fluorescent-activated cell sorting buffer to the compensation control wells.

4.7. Once the plate is centrifuged, resuspend the cell pellet and controls in 200 microliters of saponin buffer and fluorescent-activated cell sorting buffer, respectively **[1]**. Centrifuge the plate at 500 g for 6 minutes at 4 degrees Celsius **[2]** and remove the supernatant **[3-TXT]**.

4.7.1. Talent resuspending the cell pellet in 200 microliters of saponin buffer.

4.7.2. Talent centrifuging the plate at 500 g for 6 minutes at 4 degrees Celsius.

4.7.3. Talent inverting the plate to discard the supernatant. **TXT: Repeat wash 1x**

4.8. Resuspend both the cell samples and compensation controls in 200 microliters of fluorescent-activated cell sorting buffer **[1]**. Transfer the suspensions into labeled fluorescent-activated cell sorting tubes **[2-TXT]**.

4.8.1. Talent adding 200 microliters of fluorescent-activated cell sorting buffer to each well.

4.8.2. Talent transferring the contents into FACS tubes. **TXT: Store at 4 °C**

Results

5. Results

5.1. Single-cell RNA sequencing identified a distinct microglial cluster separate from other brain cell types based on transcriptional profiles **[1].**

5.1.1. LAB MEDIA: Figure 2. *Video editor: Highlight the isolated cluster labeled "Microglial cells" located near the top center of the UMAP plot*.

5.2. Differential expression analysis revealed significantly upregulated genes in microglia, including Csf1r *(C-S-F-One-R),* Fcrls *(F-C-R-L-S)*, Fyb *(F-Y-B)*, Adap2 *(A-dap-two),* and P2ry12 *(P-Two-R-Y-Twelve)* **[1].**

5.2.1. LAB MEDIA: Figure 4. *Video editor: Zoom in on the upper-right region of the volcano plot where red-labeled gene names like Csf1r and P2ry12 are clustered*.

5.3. Microglial cells were successfully isolated from live brain samples based on their distinct expression of CD45 *(C-D-Forty-five)* and CD11b *(C-D-Eleven-B)* markers **[1].**

5.3.1. LAB MEDIA: Figure 5. *Video editor: Highlight 5D*.

5.4. Distinct microglial subpopulations were identified based on combinations of activation markers, including CD80 *(C-D-Eighty)*, CD86 *(C-D-Eighty-Six)*, iNOS *(I-Nos)* **[1],** CD206 *(C-D-Two-Zero-Six)* and Arg1 *(Arg-One)* **[2],** CD86 and CD64 *(C-D-Sixty-Four)* **[3],** and CD163 with CD206 **[4].**

5.4.1. LAB MEDIA: Figure 6A. *Video editor: Highlight the gated areas for CD80, CD86, and iNOS, shown in sequence across the three panels.*

5.4.2. LAB MEDIA: Figure 6B. Video editor: Highlight the upper right quadrant in both CD206 and Arg1 dot plots.

5.4.3. LAB MEDIA: Figure 6C. *Video editor: Highlight the upper right quadrant in both CD86 and CD64 dot plots*.

5.4.4. LAB MEDIA: Figure 6D. *Video editor: Highlight the upper right quadrant in both CD163 and CD206 dot plots.*

5.5. Marker gene expression of Ptprc *(P-T-P-R-C)* and Itgam *(I-T-G-A-M)* was localized specifically to the microglial cluster in the UMAP *(U-Map)* projection, confirming the identity of these cells **[1].**

5.5.1. LAB MEDIA: Figure 7. *Video editor: Highlight the bright blue cluster labeled*

"Microglial cells" in Ptprc and Itgam expression map.

5.6. Violin plot analysis showed lower expression of anti-inflammatory markers such as *Fcgr1 (F-C-G-R-One)* and CD86 in treated microglia compared to vehicle control **[1].**

5.6.1. LAB MEDIA: Figure 8. *Video editor: Highlight the purple distributions for Fcgr1and Cd86*

Pronunciation Guide:

1. **Microglial**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/microglia
   IPA: /ˌmaɪ.kroʊˈgliː.əl/
   Phonetic Spelling: my-kroh-glee-uhl

2. **Collagenase**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/collagenase
   IPA: /kəˈlædʒəˌneɪs/
   Phonetic Spelling: kuh-la-juh-nays

3. **DNase**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/DNase
   IPA: /ˈdiːˌɛn.eɪz/
   Phonetic Spelling: dee-en-ays

4. **Parafilm**
   Pronunciation link:
   https://www.howtopronounce.com/parafilm
   IPA: /ˈpær.əˌfɪlm/
   Phonetic Spelling: pair-uh-film

5. **Myelin**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/myelin
   IPA: /ˈmaɪə.lɪn/
   Phonetic Spelling: my-uh-lin

6. **Fluorescent**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/fluorescent
   IPA: /flʊˈrɛsənt/ or /flɔːˈrɛsənt/
   Phonetic Spelling: floo-reh-suhnt

7. **Cytometry** (from Flow Cytometry)
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/cytometry
   IPA: /saɪˈtɑːmətri/
   Phonetic Spelling: sy-tah-muh-tree

8. **Saponin**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/saponin
   IPA: /ˈsæpənɪn/
   Phonetic Spelling: sah-puh-nin

9. **Paraformaldehyde**
   Pronunciation link:
   https://www.merriam-webster.com/dictionary/paraformaldehyde
   IPA: /ˌpærəfɔːrˈmældəˌhaɪd/
   Phonetic Spelling: pair-uh-for-mal-duh-hyde

10. **Transcriptomics** (from transcriptional profiles)
    Pronunciation link:
    https://www.howtopronounce.com/transcriptomics
    IPA: /ˌtrænskrɪpˈtoʊmɪks/
    Phonetic Spelling: tran-skrip-toh-miks

11. **Volcano plot** (biostatistics term)
    Pronunciation link:
    https://www.howtopronounce.com/volcano-plot
    IPA: /vɑlˈkeɪ.noʊ plɑːt/
    Phonetic Spelling: vahl-kay-noh plot