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Title: Identifying Microglia and Peripheral Infiltrating Macrophages in the Injured Spinal Cords Using Flow Cytometry

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

2. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? No

3. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? Yes, all done

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: **06/30/2025**

When you are ready to submit your video files, please contact our China Location Producer, Yuan Yue.

Current Protocol Length

Number of Steps: 19
Number of Shots: 41 (38 SC)

Introduction

1.1. **Yu-Qing Chen:** Our research identifies microglia and infiltrating macrophages in spinal cord injury to clarify their roles in neuroinflammation using optimized flow cytometry.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.4.2*

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

1.2. **Yu-Qing Chen**: Flow cytometry and colloidal silica density gradient centrifugation are key for isolating and phenotyping CNS immune cells with high specificity and efficiency.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.7.2*

What are the current experimental challenges?

1.3. **Yu-Qing Chen:** Distinguishing morphologically similar microglia and macrophages, minimizing cell loss during isolation, and ensuring marker specificity in dynamic injury microenvironments remain key hurdles.

1.3.1. What significant findings have you established in your field?INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.8.3*

What research gap are you addressing with your protocol?

1.4. **Yu-Qing Chen:** Existing methods fail to reliably separate resident microglia from infiltrating macrophages. Our marker-based approach resolves this critical limitation in spinal cord injury studies.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.2.1*

Ethics Title Card

This research has been approved by the Animal Care Ethics Committee at the Bengbu Medical University

Protocol

2. Flow Cytometric Analysis of Experimental Mouse Spinal Cord Cells

Demonstrator: Yu-Qing Chen

2.1. To begin, analyze the isotype control tube using the flow cytometer with the compatible software **[1]**. Adjust the forward scatter and side scatter voltages to position the cell population within an appropriate range **[2]**.

2.1.1. WIDE: Talent placing the isotype control tube into the flow cytometer. **NOTE**: If the authors have not provided the footage for this shot then please use the screen capture video SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 00:01-00:10

2.1.2. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 00:11-00:25.

2.2. Set up two additional graphs in the software: one with CD45-APC *(C-D-forty five-A-P-C)* on the x-axis and CD11b-PE *(C-D-eleven-B-P-E)* on the y-axis **[1]**, and another with CD68-FITC *(C-D-sixty eight-fit-cee)* on the x-axis and CCR7 APC-eFluor *(e-fluor)* 780 on the y-axis **[2]**.

2.2.1. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 00:40-00:54.

2.2.2. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 01:00-01:10.

2.3. Then, adjust the fluorescence channel voltages for CD45-APC, CD11b-PE, CD68-FITC, and CCR7 APC-eFluor 780 to position the cell population within the double-negative region marked at 10 to the power of 2 **[1]**.

2.3.1. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 01:15-01:20 and 01:40-01:48.

2.4. After confirming the optimized voltages using the isotype control tube, retain all settings **[1]**. Proceed to analyze the antibody-stained experimental tubes **[2]**.

2.4.1. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 01:50-02:00

2.4.2. Talent placing the antibody-stained experimental tubes into the flow cytometer. **NOTE**: If the authors have not provided the footage for this shot then please use

the screen capture video SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 02:01-02:10

2.5. Establish a graph with CD11b-PE on the x-axis and side scatter on the y-axis **[1]**. Set up another graph with CD68-FITC and CCR7 APC-eFluor 780 **[2]**. Using the isotype control as a reference, delineate the CD11b+ region and define it as the P1 gate **[3]**.

2.5.1. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 02:12-02:22.

2.5.2. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 02:35 -02:48.

2.5.3. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 02:50-03:06.

2.6. Select the CD68-FITC and CCR7 APC-eFluor 780 graph **[1]**. Right-click to open **Properties** and assign the F4 column to **P1** **[2]**.

2.6.1. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 03:07 -03:13

2.6.2. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 03:14 -03:31.

2.7. After acquiring the data, adjust the fluorescence compensation as needed **[1]**. Analyze and record the percentage of CD11b CD68 CCR7 positive and CD11b CD68 positive CCR7- *(C-C- R-7-negative)* cell populations **[2]**.

2.7.1. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 03:40-03:50.

2.7.2. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 04:53 -05:05.

2.8. Begin analyzing the experimental tubes using the voltage settings from the isotype control tube **[1]**.

2.8.1. Talent loading new experimental tubes into the flow cytometer and starting the next round of data collection. **NOTE**: If the authors have not provided the footage for this shot then please use the screen capture video SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 05:06 -05:16

2.9. Establish a graph with CD45-APC and CD11b-PE **[1]**, then set up two graphs with CD68-

FITC and CCR7 APC-eFluor 780 **[2]**. Acquire 50,000 events per sample, and adjust the fluorescence compensation after data collection **[3]**.

2.9.1. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4: 05:20 -05:37.

2.9.2. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4: 05:38 -05:51.

2.9.3. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4: 06:20-06:36.

2.10. In the pseudocolor plots of CD45 and CD11b, identify and analyze the regions for CD11b+ CD45− *(CD-11-B positive C-D-45-negative)* low and CD11b+ CD45 *(CD-11-B positive C-D-45)* high cells **[1]**. Then, analyze the CD68+ CCR7+ and CD68+ CCR7- *(C-C-R-7-negative)* populations within each region. Integrate all analyses to determine the percentage of M1-like and M2-like microglia and macrophages across the defined regions **[2]**.

2.10.1. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4: 06:40-07:00.

2.10.2. SCREEN:Flow-Cytometric-Analysis-of-Experimental-Mouse-Spinal-Cord-Cells.mp4 07:09-07:34.

3. Flow Cytometry Data Analysis

3.1. Launch the flow cytometry analysis software **[1]**. Drag the flow cytometry experiment files into the **All Samples** section of the interface **[2]**.

3.1.1. SCREEN: Flow-Cytometry-Data-Analysis.mp4 00:00-00:11.

3.1.2. SCREEN: Flow-Cytometry-Data-Analysis.mp4 00:12-00:35.

3.2. Double-click on the isotype control sample to open a two-dimensional dot plot **[1]**. Select **FSC-A** for the x-axis and **SSC-A** for the y-axis **[2]** and click on the **Rectangular Gate** button and use it to select the area excluding cellular debris **[3]**.

3.2.1. SCREEN: Flow-Cytometry-Data-Analysis.mp4 00:36-00:46.

3.2.2. SCREEN: Flow-Cytometry-Data-Analysis.mp4 00:47-00:58.

3.2.3. SCREEN: Flow-Cytometry-Data-Analysis.mp4 00:59-01:10.

3.3. Double-click the gated area to open a new histogram plot **[1]**. Then, select **FITC** for the x-axis and **Histogram** for the y-axis **[2]**. Click on the **Region Gate** button to define the threshold between negative and positive fluorescence signals **[3]**.

3.3.1. SCREEN: Flow-Cytometry-Data-Analysis.mp4 01:11-01:19.

3.3.2. SCREEN: Flow-Cytometry-Data-Analysis.mp4 01:20-01:31.

3.3.3. SCREEN: Flow-Cytometry-Data-Analysis.mp4 01:35-01:48.

3.4. For each of the four fluorescent antibodies, determine the threshold between negative and positive signals using the isotype control sample **[1]**. Sequentially change the x-axis fluorochrome to **PE**, **APC**, and **APC-Cy7**, while keeping the y-axis as Histogram **[2]**.

3.4.1. SCREEN: Flow-Cytometry-Data-Analysis.mp4 : 01:50-02:09.

3.4.2. SCREEN: Flow-Cytometry-Data-Analysis.mp4 02:10-02:29.

3.5. Use the **Region Gate** tool in each histogram to finalize gates for distinguishing negative and positive fluorescence signals for the four antibodies **[1]**.

3.5.1. SCREEN: Flow-Cytometry-Data-Analysis.mp4 02:32-02:48.

3.6. Next, double-click on the experimental sample to open a dot plot **[1]**. Select **FSC-A** for the x-axis and **SSC-A** for the y-axis **[2]**. Click the **Rectangular Gate** button to gate the population after removing debris **[3]**.

3.6.1. SCREEN: Flow-Cytometry-Data-Analysis.mp4 : 02:50-03:00.

3.6.2. SCREEN: Flow-Cytometry-Data-Analysis.mp4 03:01-03:11.

3.6.3. SCREEN: Flow-Cytometry-Data-Analysis.mp4 03:12-03:26.

3.7. Double-click the gated region to open a new dot plot **[1]**. Select **CD11b-PE** for the x-axis and **SSC-A** for the y-axis **[2]**. Click the **Rectangular Gate** button and use the PE threshold from the isotype control to define the CD11b positive gate **[3]**.

3.7.1. SCREEN: Flow-Cytometry-Data-Analysis.mp4 03:27-03:38.

3.7.2. SCREEN: Flow-Cytometry-Data-Analysis.mp4 03:39-03:50.

3.7.3. SCREEN: Flow-Cytometry-Data-Analysis.mp4 04:02-04:22.

3.8. Now, double-click the CD11b positive region to open another dot plot **[1]**. Select **CD68-FITC** for the x-axis and **CCR7 APC-Cy7** for the y-axis **[2]**. Click the **Cross Gate** button and use the isotype-derived FITC and APC-Cy7 thresholds to categorize cells into M1-like

and M2-like types **[3]**.

3.8.1. SCREEN: Flow-Cytometry-Data-Analysis.mp4 : 04:23-04:36.

3.8.2. SCREEN: Flow-Cytometry-Data-Analysis.mp4 04:37-04:49.

3.8.3. SCREEN: Flow-Cytometry-Data-Analysis.mp4 04:55-05:15.

Results

4. Results

4.1. The proportion of CD45high CD11b+ *(read all '+' as 'positive')* CD68+ CCR7+ cells was significantly increased in the spinal cord injury or SCI vehicle group compared to the sham group **[1]**, and CRID3 *(crid-3)* treatment did not alter this level **[2]**.

4.1.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the bar for CD45highCD11b+CD68+CCR7+ cells in the SCI (vehicle) group*.

4.1.2. LAB MEDIA: Figure 3B *Video editor: Highlight the CD45highCD11b+CD68+CCR7+ bar in the CRID3 group* .

4.2. The proportion of CD45negative low CD11b+ CD68+ CCR7+ cells was significantly higher in the SCI vehicle group compared to sham **[1]**, and was significantly reduced following CRID3 treatment **[2]**.

4.2.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the tall bar for CD45−/lowCD11b+CD68+CCR7+ in the SCI (vehicle) group*.

4.2.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the CD45−/lowCD11b+CD68+CCR7+ bar in the CRID3 group*.

4.3. The proportions of CD11b+ CD68+ CCR7+ cells were significantly increased in the SCI vehicle group compared to sham **[1]**, and significantly decreased after CRID3 treatment **[2]**.

4.3.1. LAB MEDIA: Figure 3 B. *Video editor: Highlight the increased bar for CD11b+CD68+CCR7+ in the SCI (vehicle) group*.

4.3.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the bar for CD11b+CD68+CCR7+ in the CRID3 group*.

4.4. CD11b+ CD68+ CCR7negative and CD45negative low CD11b+ CD68+ CCR7 negative cell proportions were significantly decreased in both SCI vehicle groups compared to sham **[1]**, and increased after CRID3 treatment **[2]**.

4.4.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the shorter bars for CD11b+CD68+CCR7− and CD45−/lowCD11b+CD68+CCR7− in the SCI (vehicle) group*.

4.4.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the bars for CD11b+CD68+CCR7− and CD45−/lowCD11b+CD68+CCR7− in the CRID3 group* .

Pronunciation guide:

1. **Isotype**
    - **Pronunciation link**: https://www.merriam-webster.com/medical/isotype
    - **IPA**: /ˈaɪ.səˌtaɪp/
    - **Phonetic Spelling**: eye-suh-type(merriam-webster.com)
2. **Flow Cytometry**
    - **Pronunciation link**: https://www.merriam-webster.com/dictionary/flow%20cytometry
    - **IPA**: /ˈfloʊ saɪˈtɑː.mə.tri/
    - **Phonetic Spelling**: floh sigh-tah-muh-tree(merriam-webster.com)
3. **Fluorescence**
    - **Pronunciation link**: https://www.merriam-webster.com/dictionary/fluorescence
    - **IPA**: /ˌflʊrˈɛs.ənts/
    - **Phonetic Spelling**: floor-ess-uhns(merriam-webster.com)
4. **Microglia**
    - **Pronunciation link**: https://www.merriam-webster.com/medical/microglia
    - **IPA**: /maɪˈkrɑː.gli.ə/
    - **Phonetic Spelling**: my-krah-glee-uh(merriam-webster.com)
5. **Macrophage**
    - **Pronunciation link**: https://www.merriam-webster.com/dictionary/macrophage
    - **IPA**: /ˈmæk.rəˌfeɪdʒ/
    - **Phonetic Spelling**: mak-ruh-fayj
6. **Cytometry**
    - **Pronunciation link**: https://www.merriam-webster.com/medical/cytometry
    - **IPA**: /saɪˈtɑː.mə.tri/
    - **Phonetic Spelling**: sigh-tah-muh-tree(merriam-webster.com)
7. **Cytometer**
    - **Pronunciation link**: https://www.merriam-webster.com/medical/cytometer
    - **IPA**: /saɪˈtɑː.mə.tər/
    - **Phonetic Spelling**: sigh-tah-muh-ter(merriam-webster.com)
8. **APC** (as in Antigen-Presenting Cell)
    - **Pronunciation link**: https://www.merriam-webster.com/dictionary/APC
    - **IPA**: /ˌeɪ.piːˈsiː/
    - **Phonetic Spelling**: ay-pee-see(merriam-webster.com)