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Title: Assessing the Expression of Major Histocompatibility Complex Class I on Primary Murine Hippocampal Neurons by Flow Cytometry

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Y**

If **Yes**, can you record movies/images using your own microscope camera?

Dissection scope: Swift M29TZ-SM99CL-BTW1. Inverted tissue culture microscope: Olympus CKX53

2. Software: Does the part of your protocol being filmed demonstrate software usage? **N**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Script Length
Number of shots: **50**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Sarah Lotz**: This protocol uses flow cytometry to quantitatively assess extracellular MHCI expression on primary neurons cultured from mice **[1]**.

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

REQUIRED:

1.2. **Kristen Funk**: In situ immunostaining for MHCI expression can also be performed to avoid a loss of signal due to protein tertiary structure changes, permeabilization, or denaturing conditions **[1]**.

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

OPTIONAL:

1.3. **Sarah Lotz**: In addition to directing immune responses to infections, MHCI modulates neuronal synaptic connections. However, the factors that regulate MHCI expression are still unknown **[1]**.

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

OPTIONAL:

1.4. **Kristen Funk**: The embryonic brain dissection steps take practice to master. When learning the technique, take care to practice the dissection without worrying about the time or subsequent culturing process **[1]**.

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

Ethics Title Card

1.5. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Charlotte.

Protocol

2. Embryonic Hippocampus Dissection

2.1. For isolation of the embryonic hippocampus, place the first harvested mouse embryonic brain under a stereo dissection microscope **[1-TXT]** and use two pairs of sterile Dumont number 5 forceps to pinch off the olfactory bulbs and to thoroughly pull away the meninges **[2]**.

2.1.1. WIDE: Talent placing brain under microscope **TEXT: See text for brain harvest details**

2.1.2. SCOPE: Bulbs being pinched, the meninges being pulled NOTE: This and next shot together *Videographer: Important step*

2.2. Once the meninges have been completely removed, the superior side of the cortex will open laterally to expose the hippocampus **[1]**.

2.2.1. SCOPE: Cortex being opened laterally

2.3. Use the forceps to pinch the hippocampus away from the attached cortex **[1]** and carefully transfer the isolated hippocampus to a sterile 15-milliliter conical tube containing 5 milliliters of Hibernate-E medium on ice **[2-TXT]**.

2.3.1. SCOPE: Hippocampus being pinched NOTE: This and next shot are both CAM and SCOPE *Videographer: Important step*

2.3.2. Talent placing hippocampus into tube **TEXT: Repeat for each hemisphere/brain**

3. Hippocampal Neuron Dissociation and Culture

3.1. When all of the hippocampi have been collected into a single, 15-milliliter tube **[1-TXT]**, sediment the brain tissue by centrifugation **[2-TXT]**.

3.1.1. WIDE: Talent adding brain to tube **TEXT: Cortex may be isolated and processed for cortical neuron culture**

3.1.2. Talent placing tube(s) into centrifuge **TEXT: 5 min, 1000 x g, RT**

3.2. Replace the supernatant with 0.5 milliliters of freshly prepared papain dissociation per embryo **[1-TXT]** and mix the tube several times by inversion **[2]**.

3.2.1. Shot of tissue at bottom of tube, then papain being added to tube, with papain, DNase I, and medium containers visible in frame **TEXT: See text for all solution preparation details** NOTE: This and next shot together
3.2.2. Talent inverting tube

3.3. Place the tissues at 37 degrees Celsius for 30 minutes, mixing the samples by inversion every 10 minutes **[1]**, before collecting the tissue again by centrifugation **[2-TXT]**.

3.3.1. Talent removing tube from 37 °C to invert
3.3.2. Talent placing tube into centrifuge **TEXT: 10 min, 125 x g, RT**

3.4. Replace the supernatant with an equal volume of fresh Hibernate E medium **[1]** and use a fully open, glass, fire polished Pasteur pipette to triturate the tissue 10 times **[2]**.

3.4.1. Medium being added to tube, with medium container visible in frame
3.4.2. Shot of fully open pipette, then tissue being triturated *Videographer: Important step*

3.5. After letting the tissue settle for 2 minutes, transfer the supernatant into a new 50-milliliter conical tube **[1]**.

3.5.1. Shot of settled tissue, then supernatant being transferred to new tube NOTE: 3.5.1 – 3.6.2 shot together *Videographer: Important step*

3.6. Add an equal volume of Hibernate E medium back to the tissue **[1]** and use a half-open, glass, fire polished Pasteur pipette to triturate tissue 10 times **[2]**.

3.6.1. Talent adding medium to tube, with medium container visible in frame *Videographer: Important step*
3.6.2. Shot of half open pipette, then tissue being triturated NOTE: The first 3.6.2 is slated as pipette prep *Videographer: Important step*

3.7. After letting the tissue settle for another 2 minutes, pool the supernatant in the 50-milliliter tube **[1]**.

3.7.1. Shot of settled tissue, then supernatant being transferred to new tube NOTE: This and next shot together *Videographer: Important step*

3.8. Add an equal volume of Hibernate E medium to the tissues **[1]** and triturate the tissue 10 times with a quarter-open, glass, fire polished Pasteur pipette **[2]**.

3.8.1. Medium being added to tube, with medium container visible in frame
3.8.2. Shot of quarter open pipette, then tissue being triturated

3.9. After letting the tissue settle for 2 minutes, pool the supernatant in the 50-milliliter tube **[1-TXT]**.

3.9.1. Shot of settled tissue, then supernatant being transferred to new tube
Videographer: Important step **TEXT: Discard any non-dissociated tissue**

3.10. Collect the dissociated cells in the supernatant by centrifugation **[1-TXT]** and resuspend the pellet in 5 milliliters of neuron growth medium **[2]** for counting **[3]**.

3.10.1. Talent placing tube(s) into centrifuge **TEXT: 5 min, 125 x g, RT**
3.10.2. Shot of pellet if visible, then medium being added to cells, with medium container and counter visible in frame
3.10.3. Added shot counting

3.11. Dilute the cells to a final plating density of 5 x 10^5 viable cells per milliliter of neuron growth medium **[1]** and add 1 milliliter of cells to each well of a 12-well, poly-D-lysine-coated plate **[2]**.

3.11.1. Talent adding medium to tube, with medium container visible in frame, with plate visible in frame NOTE: This and next shot together
3.11.2. Cells being added to plate

3.12. Then place the plate in the cell culture incubator **[1]**, replacing half of the medium with an equal volume of fresh medium twice a week for the lifespan of the culture **[2]**.

3.12.1. Talent placing plate into incubator NOTE: Use the second shot with Sarah
3.12.2. Medium being refreshed in well(s), with medium container visible in frame
3.12.2B Added shot: refresh

4. Flow Cytometric Major Histocompatibility Complex (MHCI) Expression Analysis

4.1. To assess the ability of the cultured neurons to express MHC (M-H-C) one, at the appropriate day of culture, replace 0.5 milliliter of supernatant in each well with 0.5 milliliters of fresh neuron growth medium supplemented with 200 units/milliliter of interferon-beta **[1]** for a 6-72-hour incubation in the cell culture incubator **[2-TXT]**.

4.1.1. WIDE: Talent adding medium to well(s), with medium and INF-beta containers visible in frame **TEXT: Control wells: medium alone**
4.1.2. Talent placing plate into incubator NOTE: Reuse 3.12.1

4.2. At the end of the incubation, wash each well one time with cold neurobasal medium without supplements **[1]** before adding 0.5 milliliters of non-supplemented cold

neurobasal media supplemented with 1 microgram/milliliter of Fc (F-C) block and 1 microgram/milliliter of fluorescence-conjugated anti-MHC one antibody to each well **[2]**.

4.2.1. Talent washing well(s), with medium container visible in frame
4.2.2. Talent adding medium to well(s), with Fc block and antibody containers visible in frame

4.3. After a 45-minute incubation at 4 degrees Celsius protected from light **[0]**, wash each well one time with cold Dulbecco's PBS **[1]**.

4.3.0 Added shot: fridge
4.3.1. Talent washing well(s), with DPBS container visible in frame NOTE: 4.3.1 – 4.4.2 together

4.4. Next, add 0.5 milliliters of room-temperature enzyme-free cell dissociation buffer to each well **[1]** and agitate to dislodge the cells **[2]**.

4.4.1. Talent adding buffer to well(s), with buffer container visible in frame
4.4.2. Plate being agitated

4.5. Confirm dissociation under an inverted tissue culture microscope **[1]** and add 0.5 milliliters of FACS (facks) buffer to each well **[2-TXT]**.

4.5.1. Talent checking dissociation
4.5.2. Talent adding buffer, with buffer container visible in frame **TEXT: FACS: fluorescence-activated cell sorting** NOTE: 4.5.2 – 4.6.2 together

4.6. Triturate the cells to disperse clumps **[1]** and transfer the entire volume from each well into individual 1.7-milliliter microcentrifuge tubes **[2]**.

4.6.1. Cells being triturated
4.6.2. Talent adding cells to tube(s)

4.7. Collect the cells by centrifugation **[1-TXT]** and resuspend the pellet in 100 microliters of fresh FACS buffer per tube **[2]**.

4.7.1. Talent adding tube(s) to centrifuge **TEXT: 5 min, 1000 x g**
4.7.2. Talent adding buffer to tube, with buffer container visible in frame NOTE: This and next shot together

4.8. Transfer each suspension into individual wells of a 96-well, U-bottom plate **[1]** and add 100 microliters of fixative reagent to each well **[2]**.

4.8.1. Talent adding cells to well(s), with tubes visible in frame
4.8.2. Talent adding fixative to well, with fixative container visible in frame

4.9. Triturate several times to avoid cell clumping **[1]** and incubate the plate for 15 minutes at room temperature protected from light **[2]**.

4.9.1. Well(s) being triturated NOTE: This and next shot together
4.9.2. Talent covering plate

4.10. At the end of the incubation, centrifuge to collect the cells at the bottom of the wells **[1-TXT]** and resuspend the pellets in 200 microliters of fresh FACS buffer per well **[2]**.

4.10.1. Talent adding plate to centrifuge **TEXT: 5 min, 500 x g, RT**
4.10.2. Talent adding buffer to well(s), with buffer container visible in frame

4.11. After centrifuging, resuspend the pellets in 100 microliters of permeabilization reagent supplemented with fluorescence-conjugated anti-neuronal nuclei antibody per well **[1]**.

4.11.1. Talent adding reagent to well(s), with reagent and antibody containers visible in frame

4.12. After mixing, incubate the plate for 20 minutes at room temperature with rocking protected from light **[1]**.

4.12.1. Talent placing plate onto rocker

4.13. At the end of the incubation, collect the cells by centrifugation three times **[1]**, resuspending the pellets in 100 microliters of fresh FACS buffer between centrifugations **[2]**.

4.13.1. Talent placing plate into centrifuge NOTE: reuse 4.10.1.
4.13.2. Talent adding buffer to well(s), with buffer container visible in frame

4.14. After the last wash, resuspend the cells in 100 microliters of 2% paraformaldehyde in FACS buffer with thorough mixing **[1-TXT]**.

4.14.1. Talent adding PFA to well(s), with PFA container visible in frame **TEXT: Optional: Store ≤1 wk at 4 °C before analysis**

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.1., 2.3., 3.4.-3.7.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?

n/a

Results

5. Results: Representative Gating Strategy and MHCI Quantification

5.1. Neurons can be identified through the sequential gating of total events **[1]** to exclude cellular debris and doublets **[2]** and by their neuronal nuclei-positivity **[3]**.

5.1.1. LAB MEDIA: Figures 1A-1C *Video Editor: please emphasize gates in Figure 1A*
5.1.2. LAB MEDIA: Figures 1A-1C *Video Editor: please emphasize gates in Figure 1B*
5.1.3. LAB MEDIA: Figures 1A-1C *Video Editor: please emphasize gate in Figure 1C/cells in gate in Figure 1C*

5.2. Neuronal nuclei-positive cells can then be further analyzed for their MHC one-positivity **[1]**.

5.2.1. LAB MEDIA: Figure 1D *Video Editor: please emphasize bracket and MHCI+ text*

5.3. From this data, the percentage of neurons positive for MHC one staining **[1]** and the median fluorescence intensity can be calculated **[2]**, revealing that, for example, interferon-beta treatment significantly upregulates the percentage and intensity of MHC one neuron expression **[3]**.

5.3.1. LAB MEDIA: Figures 1E and 1F *Video Editor: please emphasize Figure 1E*
5.3.2. LAB MEDIA: Figures 1E and 1F *Video Editor: please emphasize Figure 1F*
5.3.3. LAB MEDIA: Figures 1E and 1F *Video Editor: please emphasize red data bars*

Conclusion

6. Conclusion Interview Statements

6.1. **Kristen Funk**: With slight modifications, these methods can be used to culture other neuronal populations, like cortical neurons, or to test different cellular markers, stimulating molecules, or genetic modifications **[1]**.

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