

Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61436R1
Full Title:	Assessing the expression of major histocompatibility complex class I on primary murine hippocampal neurons by flow cytometry
Keywords:	antigen presentation; flow cytometry; hippocampus; major histocompatibility complex class I; microdissection; neuroimmune; primary neurons
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Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US\$4,200)
Please indicate the city, state/province, and country where this article will be filmed. Please do not use abbreviations.	Charlotte, North Carolina, USA

TITLE:

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

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KEYWORDS:

antigen presentation, flow cytometry, hippocampus, major histocompatibility complex class I, microdissection, neuroimmune, primary neurons

SUMMARY:

This protocol describes a method for culturing primary hippocampal neurons from embryonic mouse brain. The expression of major histocompatibility complex class I on the extracellular surface of cultured neurons is then assessed by flow cytometric analysis.

ABSTRACT:

Increasing evidence supports the hypothesis that neuro-immune interactions impact nervous system function in both homeostatic and pathologic conditions. A well-studied function of major histocompatibility complex class I (MHCI) is the presentation of cell-derived peptides to the adaptive immune system, particularly in response to infection. More recently it has been shown that the expression of MHCI molecules on neurons can modulate activity-dependent changes in the synaptic connectivity during normal development and neurologic disorders. The importance of these functions to the brain health supports the need for a sensitive assay that readily detects MHCI expression on neurons. Here we describe a method for primary culture of murine hippocampal neurons and then assessment of MHCI expression by flow cytometric analysis. Murine hippocampus is microdissected from prenatal mouse pups at the embryonic day 18. Tissue is dissociated into a single cell suspension using enzymatic and mechanical techniques, then cultured in a serum-free media that limits growth of non-neuronal cells. After 7 days in vitro, MHCI expression is stimulated by treating cultured cells pharmacologically with beta interferon. MHCI molecules are labeled in situ with a fluorescently tagged antibody, then cells are non-enzymatically dissociated into a single cell suspension. To confirm the neuronal identity, cells are fixed with paraformaldehyde, permeabilized, and labeled with a fluorescently tagged antibody that recognizes neuronal nuclear antigen NeuN. MHCI expression is then quantified on neurons by flow cytometric analysis. Neuronal cultures can easily be manipulated by either genetic modifications or pharmacologic interventions to test specific hypotheses. With slight

modifications, these methods can be used to culture other neuronal populations or to assess expression of other proteins of interest.

INTRODUCTION:

The central nervous system (CNS) was once thought to be devoid of immune surveillance, referred to as “immune privileged¹.” It is now clear that this privilege does not equate to the absolute absence of immune components, but rather, a specialized regulation that functions to limit the damage associated with immunopathology¹. In fact, communication between the CNS and the immune system is an ongoing conversation that is necessary for healthy brain development and response to infections^{2,3}.

Major histocompatibility complex class I (MHCI) molecules are polygenic and polymorphic transmembrane proteins best known for their function in presenting antigenic peptides to CD8⁺ T cells during infection⁴. Classical MHCI complexes consist of a transmembrane α -chain and an extracellular light chain, called β 2-microglobulin. The α -chain contains a polymorphic groove that binds an antigenic peptide for presentation⁵. Proper expression of MHCI on the extracellular membrane requires coordinated action of molecular chaperones at the endoplasmic reticulum to ensure proper folding of the α -chain and β 2-microglobulin along with the loading of a high affinity peptide ligand⁵. Only once MHCI complexes are assembled, are they exported from the endoplasmic reticulum to the plasma membrane⁶. Upon engagement of the cognate T cell receptor with the peptide-loaded MHCI complex, CD8⁺ T cells mediate cell killing by releasing lytic granules containing perforin and granzymes or by inducing apoptosis through binding Fas receptor on the target cell membrane⁷. Additionally, CD8⁺ T cells produce cytokines, such as gamma interferon (IFN γ) and tumor necrosis factor alpha (TNF α), which can activate antiviral mechanisms in infected cells without cytopathic effects^{8,9}. For many neurotropic viruses, CD8⁺ T cells are necessary to clear the infection from the CNS^{10–12}.

It was previously thought that neurons express MHCI only under conditions of damage, viral infection, or with in vitro cytokine stimulation. Recently, research has identified a role for neuronal expression of MHCI in synaptic remodeling and plasticity^{13,14}. Although the precise mechanisms underlying synapse regulation are not well understood, data indicates that MHCI expression level is regulated by synaptic activity^{15,16}. One hypothesis posits that neurons express paired immunoglobulin-like receptor B (PirB) presynaptically, which binds MHCI transynaptically^{13,17}. This interaction initiates a signaling cascade by PirB that opposes pathways involved in synaptic remodeling, thus reinforcing and stabilizing the synaptic connection^{17–19}. In the absence of neuronal activity, MHCI expression is decreased¹⁴, and the loss of MHCI results in defective synapse elimination and misorganized synaptic circuits^{20,21}.

The assay described here, which was adapted from Chevalier et al.⁹, uses flow cytometric analysis to quantitatively assess extracellular protein expression of MHCI on primary cultures of murine hippocampal neurons. This protocol illustrates the initial techniques for microdissecting hippocampal tissue from embryonic mouse pups. It then details processes for enzymatic and mechanical dissociation of tissue into a single cell suspension and methods for maintaining the cultures in vitro. Because they do not divide, once they are in culture, neurons must be plated in

a dish and density suitable for their experimental endpoint. Next, it outlines steps for inducing MHCI expression with beta interferon (IFN β), immunolabeling for MHCI and neuronal nuclei marker NeuN, and analyzing cells by flow cytometry. Finally, it describes procedures for assessing the flow cytometry data to identify MHCI-positive neurons and quantifying the level of the MHCI expression. Also noted in this protocol are small adjustments that can be made in order to culture cortical neurons in addition to or instead of hippocampal neurons. This protocol can be easily modified to test specific hypotheses using genetic variations or pharmacological treatments.

PROTOCOL:

All procedures were performed in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and according to the International Guiding Principles for Biomedical Research Involving Animals. The protocol was approved by the University of North Carolina at Charlotte Institutional Animal Care and Use Committee (Protocol #19-020).

1. Preparing for culture

NOTE: These procedures should be done under sterile conditions in a tissue culture-designated biosafety cabinet. See **Table 1** for media and solutions.

1.1. Sterilize all dissection tools by autoclaving in self-sealing sterilization bags.

1.2. Prepare working stocks of poly-D-lysine for treating 12-well culture dishes.

1.2.1. Dissolve poly-D-lysine in 1x borate buffer to a concentration of 100 μ g/mL (10x concentrated). Store approximately 1 mL aliquots at -20 $^{\circ}$ C until needed.

1.2.2. Dilute 10x concentrated poly-D-lysine in dPBS to 10 μ g/mL (1x) and filter sterilize.

1.2.3. Treat 12-well culture plates with 0.5 mL per well of 1x poly-D-lysine at room temperature for at least 1 h or overnight for using the next day. Ensure the volume of poly-D-lysine is enough to fully cover the bottom of the culture area.

1.2.4. Just prior to the use, remove poly-D-lysine from culture plate, rinse 3x in sterile water, and keep in sterile water until ready to plate cells. Remove all traces of liquid by a thorough aspiration prior to plating cells.

1.3. Prepare Neuron Growth Media and FACS buffer. Filter sterilize and store at 4 $^{\circ}$ C until use. Neuron Growth Media can be kept for approximately 2 wks; FACS buffer can be kept for approximately 4 wks.

2. Dissecting embryonic hippocampus

NOTE: This procedure can be performed on the benchtop as it requires the use of a stereo

microscope for the removal of meninges and microdissection of the hippocampus. Adhere to strict aseptic technique to minimize potential contamination.

2.1. Euthanize a timed-pregnant C57BL/6J female mouse at embryonic day 18 in a CO₂ chamber.

2.2. Spray the abdominal skin and fur with 70% EtOH, then pinch skin with tissue forceps and make small incision with surgical scissors. Incise the peritoneum to expose the viscera and uterus.

2.3. Grasp the uterus with standard pattern forceps, lift out of cavity, and cut the connection to the mesometrium with fine scissors. Transfer uterus with embryos to 100 mm sterile plastic culture dish placed on ice.

2.4. Using fine scissors and fine forceps, carefully cut the uterus and remove the embryonic sacs to release embryos. Transfer embryos to a new 100 mm sterile plastic culture dish containing dPBS placed on ice.

2.5. Decapitate embryonic pups using fine scissors, and transfer heads to new 100 mm sterile plastic culture dish containing Hibernate-E medium placed on ice.

2.6. To remove the brain, hold the head with a pair of fine forceps in one hand. Using curved Dumont #7 forceps in other hand, insert the tip of the forceps at the base of the skull and pinch the bone and overlying skin moving anteriorly along the midline without piercing the brain.

2.7. Using curved forceps pull apart the skin and skull to expose the brain. Sweep the curved forceps under the brain from the exposed olfactory bulb to the cerebellum to lift the brain out of the skull. Transfer the brain to a new 100 mm sterile plastic culture dish containing Hibernate-E medium placed on ice. Repeat for each brain.

NOTE: All brains may be collected in a single culture dish unless needed to be separate for experiment-specific purposes.

2.8. Under a stereo dissection microscope, working with one brain at a time and two pairs of sterile Dumont #5 forceps, pinch off olfactory bulbs and pull away meninges. Thorough removal of the meninges is necessary to avoid culture contamination by other cells and to allow further dissection.

2.9. Once meninges are properly removed, the superior side of the cortex opens laterally, which will expose the hippocampus. Using Dumont #5 forceps, pinch the hippocampus away from the attached cortex, and carefully transfer isolated hippocampus to a sterile 15 mL conical tube containing 5 mL of Hibernate-E medium on ice.

2.10. Repeat the dissection for both brain hemispheres for each embryonic mouse pup and combine all hippocampi into a single 15 mL conical tube, unless needed separately for experiment specific purposes.

2.10.1. To culture cortical neurons, the cortex may be separated from the subcortex and processed identically to hippocampal neurons.

NOTE: At this point, protocol may be paused. Store brains or dissected hippocampi in Hibernate-E medium supplemented with B27 (1%) at 4 °C for up to 1 month, though extended delay may compromise the number viable cells.

3. Dissociating and culturing hippocampal neurons

NOTE: All procedures should be performed under sterile conditions in a tissue culture designated biosafety cabinet.

3.1. Prepare 0.5 mL of papain dissociation solution per embryo for hippocampal dissociation. The volume of dissociation solution needed will vary depending on the number of embryonic brains being dissected.

NOTE: For cortical neuron culture, prepare 1.0 mL of papain solution per embryo.

3.1.1. Dissolve 20 µL of papain suspension per 1 mL of the Hibernate E medium by vortexing for approximately 3 min.

3.1.2. After papain is dissolved, add 1 µL of DNase I per 1 mL of the solution. Do not vortex the solution after DNase I has been added as this can deactivate the enzyme.

3.2. Centrifuge 15 mL conical tube containing hippocampal tissue (step 2.10) at 1,000 x g for 5 min. Remove the supernatant and add papain solution. Resuspend the tissue by inverting several times. Incubate at 37 °C for 30 min, inverting to resuspend the tissue every 10 min.

3.2.1. If culturing cortical neurons, transfer all cortices to a new 100 mm sterile culture dish, carefully aspirate media from plate, and mince tissue with fine scissors or razor blade. Add papain solution to the culture dish and transfer cortical tissue with papain solution to 15 mL conical tube using a sterile transfer pipette. Incubate tissue and enzyme solution at 37 °C for 30 min, agitating every 10 min.

3.3. During 30 min incubation, prepare 3 fire-polished glass Pasteur pipettes: 1 fully open, 1 half open, and 1 quarter open.

3.4. After the 30 min incubation, centrifuge the 15 mL conical tube containing digested brain tissues at 125 x g for 10 min. Remove the enzyme solution and replace with an equal volume of fresh Hibernate E medium.

3.5. Using fully open Pasteur pipette, triturate the tissue 10x. Let the tissue settle for 2 min, then transfer the supernatant cell suspension to a sterile 50 mL conical tube. Add an equal volume of

Hibernate E medium back to the tissue.

3.6. Using half open Pasteur pipette, triturate tissue 10x. Let the tissue settle for 2 min, then transfer the supernatant cell suspension to the same 50 mL conical tube as in the previous step. Add an equal volume of Hibernate E medium back to the tissue.

3.5. Using quarter open Pasteur pipette, triturate tissue 10x. Let the tissue settle for 2 min, then transfer the supernatant cell suspension again to the same 50 mL conical as in previous steps. Discard any remaining tissue that hasn't dissociated.

3.6. Centrifuge the 50 mL conical tube containing the supernatant from the cell suspension at $125 \times g$ for 5 min. If not already done, wash poly-D-lysine-coated plates with sterile water and remove all traces of liquid by thorough aspiration during centrifugation.

3.7. After centrifugation, discard the supernatant and resuspend the cell pellet in 5 mL of Neuron Growth Media. Count live cells using trypan blue and hemocytometer.

3.7.1. If culturing cortical neurons, add at least 10 mL of Neuron Growth Media for less dense cells and more accurate counting.

3.8. Dilute cells with Neuron Growth Media for a final plating density of 5×10^5 viable cells per mL and add 1 mL of diluted cell suspension to each well of a 12 well plate. Maintain neurons at 37°C with 5% CO_2 .

NOTE: Typically, the hippocampi from 1 embryonic mouse brain, i.e., 2 hippocampi, yield approximately 1×10^6 viable cells. This number is provided for experiment-planning purposes only and should not be considered a substitution for counting cells for each culture.

3.9. Change the growth media the day after culture by removing half of the volume of media from each well, i.e., 0.5 mL, and add an equal volume of fresh media to the side of the well to avoid disrupting the cultured cells. Complete half media changes twice weekly for the lifespan of the culture.

4. Assessing MHCI expression by flow cytometry

4.1. Prepare treatment by diluting Interferon beta ($\text{IFN}\beta$) or an equal volume of diluent in Neuron Growth Media. Since media change will be a half-volume change, prepare treatment media with 2x concentrated $\text{IFN}\beta$. i.e., to treat 3 wells of a 12 well plate with 100 U/mL of $\text{IFN}\beta$, prepare 1.5 mL with 200 U/mL $\text{IFN}\beta$ or an equal volume of $\text{IFN}\beta$ diluent for media-treated controls.

4.2. Remove 0.5 mL from each well of neurons and add 0.5 mL of Neuron Growth Media with or without $\text{IFN}\beta$ to each well. Incubate in normal growth conditions (37°C , 5% CO_2) for 6-72 h, depending on experimental conditions and signal optimization.

265 4.3. Following treatment time, remove all culture media and gently wash once in cold Neurobasal
266 media lacking supplements (B27, L-glutamine, Pen-Strep).

267
268 4.4. Add 0.5 mL of non-supplemented cold Neurobasal media containing 1 µg/mL of Fc block and
269 1 µg/mL of fluorescent conjugated anti-MHCI antibody to each well. Incubate at 4 °C protected
270 from light for 45 min.

271
272 4.5. Remove antibody-containing media and carefully wash once in cold dPBS. Add 0.5 mL room-
273 temperature enzyme-free cell dissociation buffer to each well and agitate to dislodge cells. Watch
274 for dissociation using inverted tissue culture microscope.

275
276 4.6. Once cells are detached from culture dish, add 0.5 mL of FACS buffer to each well, triturate
277 cells to disperse clumps, and transfer total volume of each well to individual 1.7 mL
278 microcentrifuge tubes.

279
280 4.7. Centrifuge at 1,000 x *g* for 5 min. Remove the supernatant, resuspend the cell pellet in 100
281 µL of FACS buffer, and transfer total volume of each tube to individual wells of a 96 well U-bottom
282 plate.

283
284 4.8. Add 100 µL of fixative reagent to each well. Triturate several times to avoid cells clumping
285 and incubate for 15 min at room temperature protected from light.

286
287 4.9. Centrifuge at 500 x *g* for 5 min. Remove the supernatant and resuspend in 200 µL of FACS
288 buffer.

289
290 4.10. Centrifuge at 500 x *g* for 5 min. Remove the supernatant and resuspend in 100 µL of
291 permeabilization reagent containing fluorescent-conjugated anti-NeuN antibody (1:100 dilution)
292 to each well. Mix well, then incubate at room temperature protected from light with rocking for
293 20 min.

294
295 4.11. After incubation, centrifuge at 500 x *g* for 5 min. Remove the supernatant and resuspend
296 in 100 µL of FACS buffer. Repeat 2x.

297
298 4.12. After the final wash, resuspend cells in 100 µL of 2% paraformaldehyde diluted in FACS
299 buffer. Mix well to prevent cells from clumping. Store at 4 °C protected from light until ready to
300 read on a flow cytometer. Read samples as soon as possible, within 1 week.

301 302 5. Quantifying and evaluating data

303
304 5.1. Measure the compensation controls for each fluorescent dye and correct any spectral
305 overlaps. Ideal compensation controls include cultured neurons that are unstained, stained with
306 anti-MHCI only, and stained with anti-NeuN only.

307
308 5.2. If possible, record 100,000 events for each sample (at least 10,000) and save as FCS files.

5.3. To analyze data, using appropriate analysis software, set up sequential gates, as depicted in **Figure 1 A-C** to select for NeuN-positive neurons.

5.3.1. Plot SSC-A (log) vs FSC-A (linear). Draw a gate on the cellular population (P1) to eliminate cellular debris from the analysis.

5.3.2. Within P1 cellular population, plot FSC-H (linear) vs FSC-A (linear). Draw a gate on single cell population.

5.3.3. Within the single cell population, plot SSC-A (log) vs NeuN (log). Draw a gate on NeuN-positive population using unstained or MHCI-only stained cells as a guide.

5.3.4. Plot a histogram of MHCI fluorescence with cellular events normalized to mode on y-axis. Using unstained or NeuN-only stained neurons as a guide, draw a horizontal gate to quantify the percent neurons positive for MHCI.

5.3.5. Export the percent cells positive for MHCI, as well as the median fluorescence intensity (MFI) for MHCI on the NeuN-positive population for statistical evaluation and graphical drawing.

REPRESENTATIVE RESULTS:

Using the procedure presented here, hippocampal tissue was dissected from prenatal mouse pups at the embryonic day 18. The tissue was dissociated into a single cell suspension using enzymatic and mechanical methods, then cultured in 12 well plates that were pre-treated with poly-D-lysine. After 7 days in vitro, cells were treated with 100 U/mL of IFN β or media only for 72 h, which stimulated the expression of MHCI. Neurons were stained in situ for MHCI before being non-enzymatically dissociated into a single cell suspension. Neurons were fixed and permeabilized, then stained intracellularly for neuronal nuclei marker NeuN. Samples were assessed by flow cytometry and data were analyzed using associated software. Neurons were identified through sequential gating of the total events to exclude cellular debris and doublets (**Figure 1A,B**). Neurons were definitively identified by NeuN-positivity (**Figure 1C**). NeuN⁺ cells were further analyzed for MHCI-positivity by plotting cells on a histogram with the number of cells normalized to the mode on the y-axis and MHCI fluorescence on the x-axis. An MHCI⁺ gate was drawn at the point where positive and negative peaks diverged (**Figure 1D**). From this, the percent neurons positive for MHCI staining (**Figure 1E**) and the median fluorescence intensity (MFI; **Figure 1F**) were calculated. Results show that IFN β treatment significantly upregulated the percentage of neurons positive for extracellular staining of MHCI, as well as the level of expression, as indicated by MFI. Statistical analysis and graphical representation were done using commercially available statistical software.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative gating strategy and MHCI quantification. Primary hippocampal neurons were treated with 100 U/mL of IFN β or media only. After 72 h, neurons were stained

extracellularly with Pacific Blue-conjugated MHCI (1 $\mu\text{g/mL}$ H2-K^b), then intracellularly labeled with PE-conjugated NeuN (1:100 dilution). Cellular fluorescence was assessed by flow cytometry, and data was analyzed. (A) Total events were plotted as SSC-A (log) vs FSC-A (linear), and cells (P1) were gated to exclude debris. (B) Within the P1 population, cells were plotted as FSC-H (linear) vs FSC-A (linear) to gate the single cell population. (C) Within the single cell population, cells were plotted FSC-H (log) vs NeuN-PE (log). NeuN⁺ cells were gated to identify neurons. (D) Within the neuron population, cells were plotted on a histogram with MHCI-PacBlu on the x-axis and cell numbers normalized to mode on the y-axis. A horizontal gate was drawn to quantify the percent of neurons positive for MHCI staining. (E) Quantification of percent MHCI⁺ of NeuN⁺ cells in media only and IFN β -treated neurons. (F) Quantification of median fluorescence intensity (MFI) of MHCI on NeuN⁺ cells in media (black) and IFN β -treated (red) neurons. Statistical significance was calculated by unpaired t test. **, P < 0.01.

Table 1: Media and solutions.

DISCUSSION:

This protocol describes the dissection and culture of primary hippocampal neurons from prenatal mouse pups at embryonic day 18. The use of primary neurons cultured from rodents is one of the most fundamental methodologies developed in modern neurobiology²². Although immortalized cell lines can model certain aspects of neurons, their nature as tumor-derived cells, failure to develop defined axons, and continued cell division raises doubts whether they faithfully recapitulate properties of post-mitotic neurons in vivo²³. Another alternative to primary neurons is the use of human induced pluripotent stem cells (HiPSCs). The technology for using HiPSCs, especially those that are patient-derived, has advanced rapidly in recent years²⁴. However, there are still limitations to working with HiPSCs including variability between cell lines, lack of functional maturity, and differences in epigenetic profiles²⁵. Although there are also limitations to working with the reductionist model of primary rodent neurons, cultured neurons retain the post-mitotic nature of neurons in vivo. Also, the expansive molecular biology tools and genetic modifications available for mice favors the use of primary neurons over HiPSCs for many applications, and mouse studies can be easily translated to the more complex in vivo organism without losing the experimental genetic system. For these reasons, many researchers use primary rodent neurons to verify key aspects, if not the bulk, of their research.

For certain assays, neurons may be analyzed directly following isolation from the brain ex vivo. This is particularly desirable for experiments involving adult mice that can be subjected to specific experimental conditions or that may depend on interactions of multiple cell types; however, there are several issues that limit the type of analyses that can be done. It is technically challenging to prepare a single cell suspension of neurons from the brains of adult mice because neurons are uniquely interconnected and ensheathed by myelin²⁶. Non-enzymatic methods of tissue trituration are inefficient at dissociating the tissue and cause cell death, whereas enzymatic preparations often cleave cell surface antigens²⁷. Furthermore, while myelin is largely absent from embryonic mice, it comprises about 20% of the adult brain, and can impair viable cell isolation and impede flow cytometry analysis²⁸. Many of the techniques that have been developed ultimately strip neurons of their cytosol and leave small, rounded cell bodies that

consist primarily of nuclei²⁹. Although this is acceptable for some analyses, this is not appropriate for quantifying cytoplasmic or extracellular protein expression. Furthermore, the reductionist cell culture system allows testing specific mechanistic questions on a shorter time scale than is frequently possible with an in vivo system.

Also described in this protocol are methods for stimulating MHCI expression pharmacologically with IFN β , and the quantification of extracellular MHCI expression by flow cytometry. Stimulation by IFN β is a useful positive control for testing other experimental conditions, but it may be noted that IFN γ and kainic acid can also stimulate MHCI expression in neurons^{9,30}, while tetrodotoxin decreases MHCI expression¹⁴. Previous methods for detecting MHCI expression relied on in situ hybridization and immunohistochemical analysis^{14,15,20,31}. While mRNA-based assays, such as in situ hybridization and qRT-PCR, can determine the spatiotemporal localization, cell type specificity, and levels of gene transcription, these assays cannot assess protein translation or transport to the plasma membrane. Immunohistochemical and western blot analysis can determine differences in protein expression and potentially cellular localization but can be difficult to accurately quantify. Furthermore, many MHCI antibodies recognize the complex's tertiary structure, and are highly sensitive to conformational changes. Thus permeabilization or denaturing conditions result in loss of MHCI immunoreactivity³². The method presented here uses in situ immunostaining for MHCI, which allows for recognition of the protein by the antibody in its native conformation, followed by fixation and permeabilization methods.

With slight modifications, the methods described here can be used to culture other neuronal populations or to assess expression of other extracellular proteins of interest. Noted in this protocol are easy modifications that can be made in order to culture cortical neurons, but the methods described here may also be used to culture other neuronal populations, such as striatal neurons³³. Furthermore, although this protocol specifies immunostaining of MHCI and NeuN, other cellular markers can be identified in a similar manner. In general, extracellular markers can be treated like MHCI and intracellular markers can be treated like NeuN. However, it should be noted that during the cellular dissociation step, axonal projections are severed from the soma. Because the gating strategy defined here screens out cellular debris and focuses on neuronal nuclei marker NeuN, proteins that are expressed exclusively in axonal projections may not be detected.

Until recently, neurons were thought to express MHCI only in response to damage, infection, or in vitro cytokine stimulation in order to engage cytotoxic CD8⁺ T cells⁹. New research has elucidated another function of MHCI in regulating synaptic connections during development¹³. The protocol described here uses IFN β to stimulate MHCI expression in wildtype cultured neurons, but similar methods may be used with a variety of cellular stimuli or genetic modifications to test specific hypotheses. This method will enable researchers to investigate the molecular mechanisms that regulate MHCI expression, which will improve understanding of the dichotomous role of MHCI on these two distinct cellular functions.

ACKNOWLEDGMENTS:

This work was supported by NIA R00 AG053412 (KEF).

DISCLOSURES:

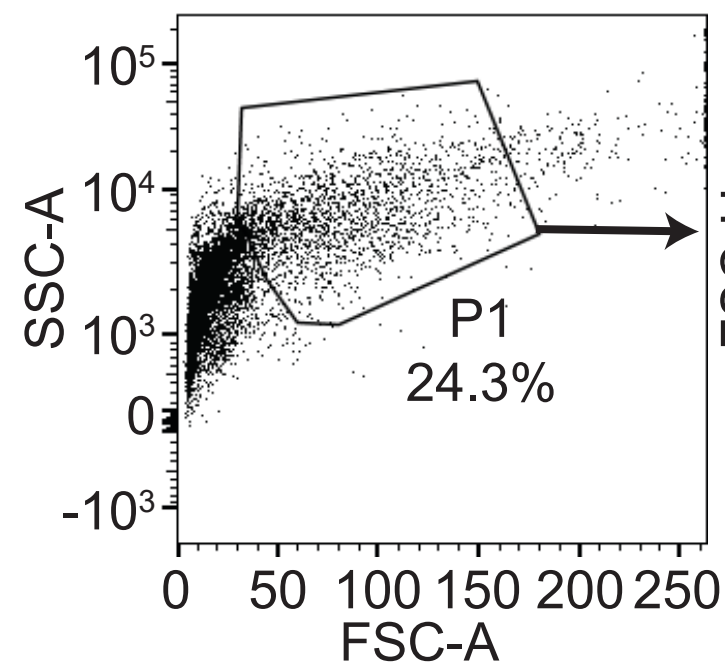
The authors have nothing to disclose.

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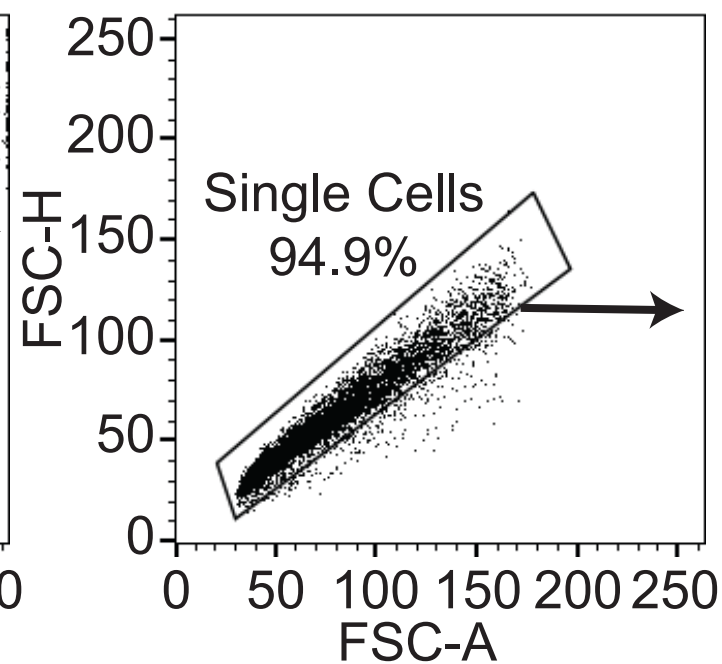
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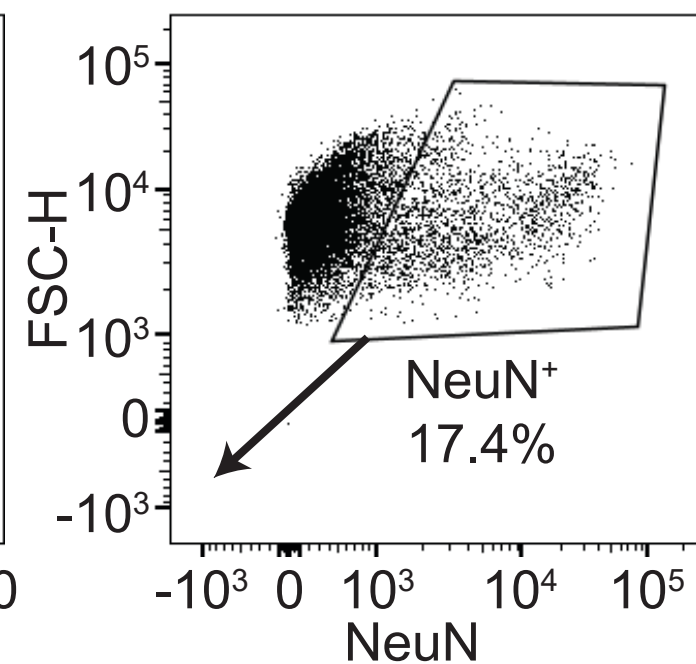
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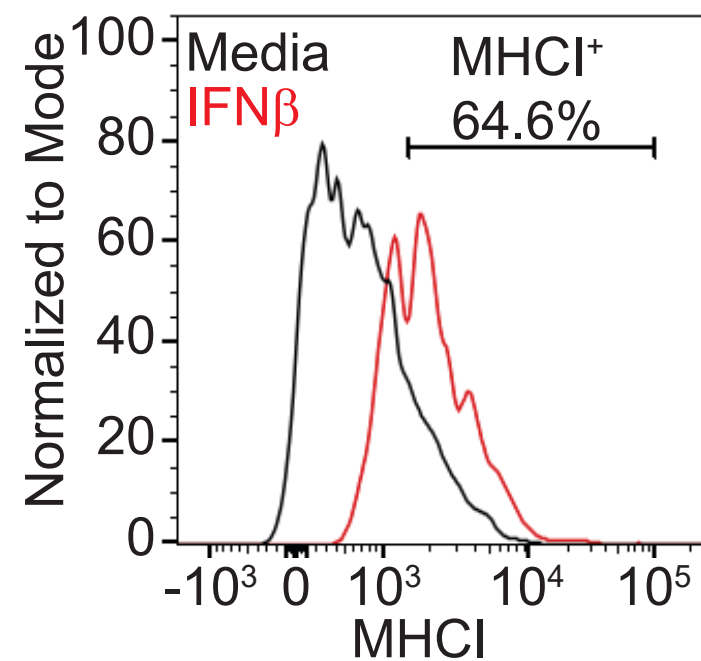
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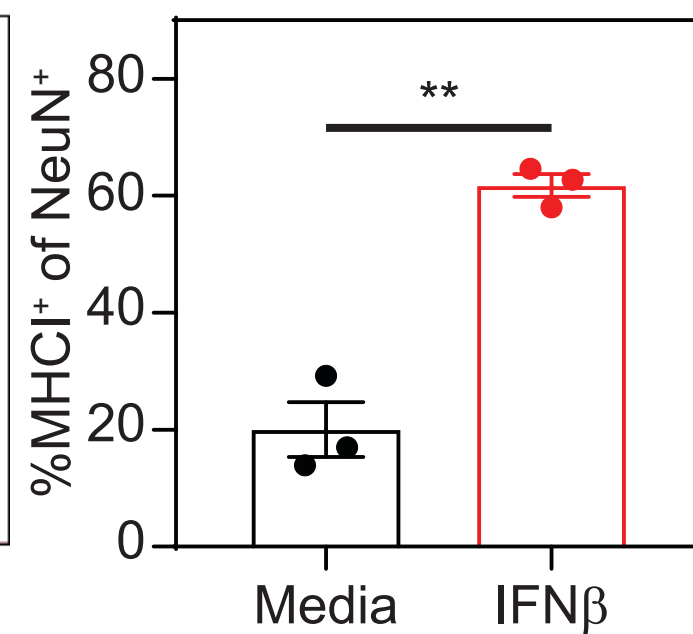
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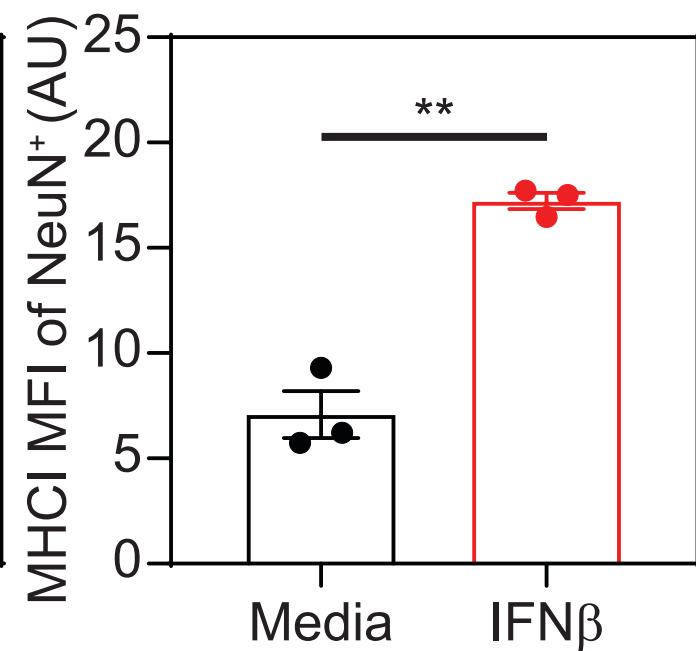


Table 1. Media and Solutions

Neuron Growth Media (50 ml)			
Reagent	Final Concentration	Stock Concentration	For 50 ml
B27 supplement	2%	100%	1.0 ml
L-glutamine	2 mM	200 mM	0.5 ml
Penicillin-Streptomycin	100 U/ml	10,000 U/ml	0.5 ml
Neurobasal media			48.0 ml

FACS buffer (500 ml)			
Reagent	Final Concentration	Stock Concentration	For 500 ml
Fetal Bovine Serum	2%	100%	10 ml
EDTA	1 mM	500 mM	1 ml
dPBS			489 ml

Papain Dissociation Solution			
Reagent	Final Concentration	Stock Concentration	For 1 ml
Papain Suspension	20 U/ml	1000 U/ml	0.020 ml
DNase I	2.5 U/ml	2500 U/ml	0.001 ml
Hibernate-E			1.0 ml

Note: The volume of Papain Dissociation Solution needed will vary depending on the number of embryonic brains being dissected. Prepare 0.5 ml per brain for hippocampal neurons or 1.0 ml per brain for cortical neurons.

Note: Prepare dissociation solution by vortexing papain suspension in Hibernate-E for about 3 min. After papain is thoroughly dissolved, add DNase I. Do not vortex DNase I.

Name of Material/Equipment	Company	Catalog Number
1.5 ml Microcentrifuge Tubes	Fisher	05-408-129
100 mm sterile culture dish	Fisher	FB012924
15 ml Centrifuge Tubes	Genesee	21-103
50 ml centrifuge tube	Genesee	21-108
500 mM EDTA	Invitrogen	AM9260G
70% Ethanol	Fisher	BP82031Gal
96 well U-bottom plate	Genesee	25-221
B27	Gibco	17504044
Borate Buffer	Thermo Scientific	28341
Borosilicate Glass Pasteur Pipette	Fisher	13 678 20C
Cell Dissociation Buffer	Gibco	13 151 014
DNase I	Thermo	90083
dPBS	Gibco	14190-235
Dumont #5 Forceps	Fine Science Tools	91150-20
Dumont #7 Forceps	Fine Science Tools	91197-00
Fetal Bovine Serum	Gibco	26140079
Fine Forceps	Fine Science Tools	91113-10
Fine Scissors	Fine Science Tools	91460-11
Fix and Perm Cell Permeabilization Kit	Invitrogen	GAS003
Glutamax	Gibco	35050061
Hibernate-E Medium	Gibco	A1247601
Instant Sealing Sterilization Pouches	Fisher	181254
Interferon Beta	PBL Assay Science	12405-1
Milli-Mark Anti-NeuN-PE Antibody, clone A60	MilliporeSigma	FCMAB317PE
Neurobasal Media	Gibco	21103049
Pacific Blue anti-mouse H-2Kb Antibody	BioLegend	116513
Papain Solution	Worthington	LS003126
Paraformaldehyde	Electron Microscopy Sciences	15714
Penicillin-Streptomycin	Gibco	15140122
Poly-D-Lysine	Sigma	P7280
Standard Pattern Forceps	Fine Science Tools	91100-12

Stereo dissection microscope	Swift	M29TZ-SM99CL-BTW1
Surgical Scissors	Fine Science Tools	91401-10
Transfer Pipets	Corning	357575
TruStain FcX (anti-mouse CD16/32) Antibody	BioLegend	101319
Trypan Blue	Sigma	T8154-100ML

Comments/Description

Laboratory Supplies
Tissue Culture Supplies
Laboratory Supplies
Laboratory Supplies
FACS Buffer Reagent
Tissue Culture Supplies
Flow Cytometry Supplies
Neuron Culture Reagent
Tissue Culture Supplies
Laboratory Supplies
Flow Cytometry Supplies
Dissociation Solution Reagent
Tissue Culture Supplies
Dissection Tool
Dissection Tool
Tissue Culture Supplies
Dissection Tool
Dissection Tool
Flow Cytometry Supplies
Neuron Culture Reagent
Neuron Culture Reagent
Tissue Culture Supplies
Flow Cytometry Supplies
Flow Cytometry Antibody
Neuron Culture Reagent
Flow Cytometry Antibody
Dissociation Solution Reagent
Fixative
Neuron Culture Reagent
Neuron Culture Reagent
Dissection Tool

Dissection Tool

Dissection Tool

Laboratory Supplies

Flow Cytometry Antibody

Tissue Culture Supplies

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April 7, 2020

Journal of Visualized Experiments
1 Alewife Center #200
Cambridge, MA 02140

Dear Editors Dr. Jewhurst and Dr. Cruz-Orengo,

Thank you for the opportunity to revise our manuscript for resubmission to the Journal of Visualized experiments. Below is a point-by-point response to the reviewers. We hope that it is now suitable for publication.

1. All three reviewers had concerns with the title.

The title has been adjusted to better reflect the true intention of the described method. The title is now: "Assessing expression of major histocompatibility complex class I on primary murine hippocampal neurons by flow cytometry"

2. Editor suggests to include detailed steps for using flow cytometer and analyzing with software.

We believe that it is beyond the scope of this methods article to detail the proper use of flow cytometer and analysis software. Every flow cytometer is different and users should receive training from their institution on its use. As for the software, although many researchers use FlowJo software, not all do, and there is no reason that data cannot be analyzed using other software. Furthermore, FlowJo software is continuously updated and may differ between MacOS and Windows systems or depending on user preset preferences. For these reasons, we request to decline the explicit detailed instructions on use of the flow cytometer and its analysis software.

3. Reviewer 2 suggested that we incorporate discussion of tetramer staining.

We understand the reviewer's perspective; however, we are concerned that this may introduce confusion. Discussion of tetramer staining will require discussion of T cell maturation, the T cell receptor, and specific antigens. While these topics are loosely related to MHCI, their understanding is not required for the method described, and these points would be better discussed in a more thorough review type article.

4. Reviewer 2 suggested we discuss neuron proliferation *in vitro* and the benefits of analyzing cells *in vitro* vs *ex vivo*.

We have addressed these points in the discussion section.

5. Reviewer 3 suggested that we discuss neuronal surface staining to eliminate the need intracellular staining.

While we agree that this would simplify the protocol, we are unfamiliar with any such markers, and NeuN remains the standard for this type of assay. In the discussion section, we acknowledge that other proteins may

be used and outline where they would fit into the protocol. There may, in fact, be extracellular neuronal markers that would work in this assay; however, we are hesitant to either endorse or reject any specific marker that we have not tested ourselves.

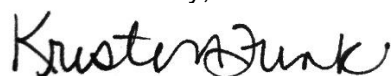
6. Reviewer 3 suggested more discussion of the expression of MHCI without IFN β stimulation.

We have included explicit discussion of the use of IFN β as a positive control as well as more references to literature regarding both positive and negative regulation of MHCI on neurons.

The following recommended edits have been corrected in the text.

1. Spacing between substeps and notes.
2. Email address has been added for co-author.
3. Media and solution preparations are being included in a separate table.
4. All mentions of “should be” have been re-written in imperative tense.
5. The strain of mice used has been indicated in step 2.1.
6. Step 2.9 has been separated into steps 2.9 and 2.10.
7. Antibody dilution is indicated as 1 μ g/ml.
8. Fix/Perm reagents have been referred to as “fixative reagent” and “permeabilization reagent.”
9. The highlighted portion of the manuscript has been confirmed to be less than 2.75 pages.
10. No portion of the included figure has been published previously.
11. Trademark (™) and registered (®) symbols have been removed from the table of equipment and materials, and the table has been sorted alphabetically.
12. We have changed all references of MHCI when unabbreviated to “major histocompatibility complex class I” for consistency.
13. Sentence in line 65-68 has been restructured for clarity.
14. The 4th paragraph in the introduction has been integrated into paragraph 2 for better flow.
15. We have re-worded the description of the assay in line 89.
16. We have changed the “mock” control to “media” control to better represent the nature of the control samples.
17. We have specified the amount of Hibernate-E that should be in the 15 ml tube.
18. We have changed “meninge” to “meninges.”
19. We have edited “CO2” to “CO₂.”

Yours sincerely,



Kristen Funk, PhD