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

Kristen E. Funk¹, Sarah K. Lotz¹

¹Department of Biological Sciences, University of North Carolina at Charlotte

Correspondence to: Kristen E. Funk at kfunk@uncc.edu

URL: https://www.jove.com/video/61436
DOI: 10.3791/61436

Keywords: Immunology and Infection, Issue 159, antigen presentation, flow cytometry, hippocampus, major histocompatibility complex class I, microdissection, neuroimmune, primary neurons

Date Published: 5/19/2020

Citation: Funk, K.E., Lotz, S.K. Assessing the Expression of Major Histocompatibility Complex Class I on Primary Murine Hippocampal Neurons by Flow Cytometry. J. Vis. Exp. (159), e61436, doi:10.3791/61436 (2020).

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 that recognizes neuronal nuclear antigen NeuN. MHCI 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 privileged”. 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 immunopathology. 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.

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 infection. Classical MHCI complexes consist of a transmembrane α-chain and an extracellular light chain, called β2-microglobulin. The α-chain contains a polymorphic groove that binds an antigenic peptide for presentation. 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 β2-microglobulin along with the loading of a high affinity peptide ligand. Only once MHCI complexes are assembled, are they exported from the endoplasmic reticulum to the plasma membrane. 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 membrane. Additionally, CD8⁺ T cells produce cytokines, such as gamma interferon (IFNγ) and tumor necrosis factor alpha (TNFα), which can activate antiviral mechanisms in infected cells without cytopathic effects. For many neurotropic viruses, CD8⁺ T cells are necessary to clear the infection from the CNS.

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

The assay described here, which was adapted from Chevalier et al., 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 (IFNβ),
immunolabeling for MHC1 and neuronal nuclei marker NeuN, and analyzing cells by flow cytometry. Finally, it describes procedures for assessing
the flow cytometry data to identify MHC1-positive neurons and quantifying the level of the MHC1 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. Sterilize all dissection tools by autoclaving in self-sealing sterilization bags.
2. Prepare working stocks of poly-D-lysine for treating 12-well culture dishes.
   1. Dissolve poly-D-lysine in 1x borate buffer to a concentration of 100 µg/mL (10x concentrated). Store approximately 1 mL aliquots at -20
      ºC until needed.
   2. Dilute 10x concentrated poly-D-lysine in dPBS to 10 µg/mL (1x) and filter sterilize.
   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.
   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.
3. Prepare Neuron Growth Media and FACS buffer. Filter sterilize and store at 4 ºC 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.

1. Euthanize a timed-pregnant C57BL/6J female mouse at embryonic day 18 in a CO2 chamber.
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.
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.
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.
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.
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.
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.
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.
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.
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.
   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
   (2%) at 4 ºC for up to 1 month, though extended delay may compromise the number of viable cells.

#### 3. Dissociating and culturing hippocampal neurons

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

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.
5. Quantifying and evaluating data

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

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

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

4. Plot SSC-A (log) vs FSC-A (linear). Draw a gate on the cellular population (P1) to eliminate cellular debris from the analysis.
2. Within P1 cellular population, plot FSC-H (linear) vs FSC-A (linear). Draw a gate on single cell population.
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.
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. 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 IFNβ 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 IFNβ 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 1: Representative gating strategy and MHCI quantification.**

Primary hippocampal neurons were treated with 100 U/mL of IFNβ or media only. After 72 h, neurons were stained extracellularly with Pacific Blue-conjugated MHCI (1 μg/mL H2-Kb), 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 SSC-A (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 IFNβ-treated neurons. (F) Quantification of median fluorescence intensity (MFI) of MHCI on NeuN+ cells in media (black) and IFNβ-treated (red) neurons. Statistical significance was calculated by unpaired t test. **, P < 0.01. Please click here to view a larger version of this figure.
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 neurobiology. 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 vivo. Another alternative to primary neurons is the use of human induced pluripotent stem cells (iPSCs). The technology for using iPSCs, especially those that are patient-derived, has advanced rapidly in recent years. However, there are still limitations to working with iPSCs including variability between cell lines, lack of functional maturity, and differences in epigenetic profiles. 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 iPSCs 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 myelin. 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 analysis. Many of the techniques that have been developed ultimately strip neurons of their cytosol and leave small, rounded cell bodies that consist primarily of nuclei. For these reasons, many researchers use primary rodent neurons to verify key aspects, if not the bulk, of their research.

Also described in this protocol are methods for stimulating MHCI expression pharmacologically with IFNβ, and the quantification of extracellular MHCI expression by flow cytometry. Stimulation by IFNβ is a useful positive control for testing other experimental conditions, but it may be noted that IFNγ and kainic acid can also stimulate MHCI expression in neurons, while tetrodotoxin decreases MHCI expression. Previous methods for detecting MHCI expression relied on in situ hybridization and immunohistochemical analysis. 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 immunoreactivity. 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 neurons. 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 cells. New research has elucidated another function of MHCI in regulating synaptic connections during development. The protocol described here uses IFNβ 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.

Disclosures

The authors have nothing to disclose.

Acknowledgments

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

References

1. Galea, I., Bechmann, I., Perry, V. H. What is immune privilege (not)? Trends in Immunology. 28 (1), 12–18 (2007).


