Dissociation of Mouse Neural Tissue for Single-Cell Western Analysis

Introduction

The mouse brain is a highly complex organ divided into several, differing regions. Within each region of the brain are various cell subtypes that perform distinct functions, but also interact with each other. Differentiating between these cell subtypes is essential to uncovering the different roles they play. Proteins play a particularly important role in defining cell identity and function including driving neurotransmitter transport, synapse formation and numerous other critical neural functions. As such, single-cell protein analysis methods are required to more clearly understand the composition of cells that make up the various regions of the brain and further elucidate their function.

The Milo Single-Cell Western system can analyze multiplexed protein expression patterns in dissociated tissues to identify and quantify cell subtypes contained in heterogeneous tissues. In this application note, we describe and characterize a protocol to successfully dissociate mouse neural tissue microsurgically dissected from combined cortex, ventricular zone, and hippocampus regions of E18 mice into single cells. Using a multiplexed Single-Cell Western assay to analyze protein expression in the dissociated single-cells, we identify 3 subpopulations of cells within the tissue: astrocytes, radial glia, and immature neurons based on expression of their respective phenotypic protein markers: GFAP, Pax6, and β-III tubulin (**Figure 1**). Cell subpopulations that are single- or multi-positive for each marker are quantified to demonstrate how cell subtypes can be identified and quantified based on multiplexed protein expression patterns using Single-Cell Western technology.

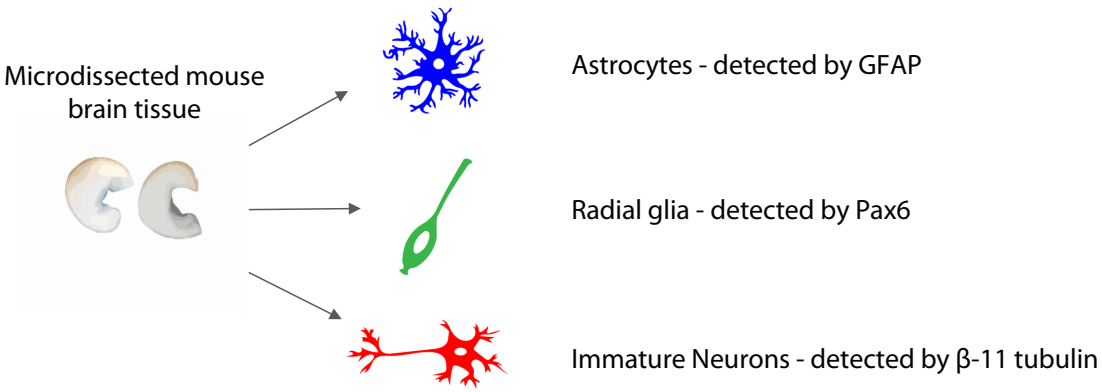

FIGURE 1. E18 mouse combined cortex, ventricular zone, hippocampus tissue composition. Immature neurons constitute the largest percentage of cells while astrocytes and radial glia are also present.

Materials

TISSUE AND TISSUE DISSOCIATION

- E18 Mouse Combined Cortex, Hippocampus and Ventricular Zone tissue from BrainBits LLC (PN C57EHCV).
- One pair of mouse tissue in 2 ml Hibernate EB media (HEB; Hibernate E/B27/GlutaMAX)
- One 12 mL tube of NbActiv1 media for primary neuron cultures (Neurobasal/B27/GlutaMAX medium)
- Papain/HE (Papain plus 5mL He-Ca) from BrainBits (PN PAP/HE)
- Sterile 9" Silanized Glass Pasteur Pipette from BrainBits (PN FPP)
- Low Retention Filter LTS Tips, Sterilized, Wide-Bore from Rainin (PN 30389218)
- Low Retention Filter LTS Tips, Sterilized from Rainin (PN 30389213)
- MACS SmartStrainers, 30 µm from Miltenyi (PN 130-098-458)

REAGENTS AND ANTIBODIES

- Mouse monoclonal β-tubulin III antibody from Sigma-Aldrich (PN T8578)
- Sheep polyclonal Pax6 antibody from R&D Systems (PN AF8150)
- Rabbit monoclonal GFAP antibody from Cell Signaling Technology (PN 80788)
- Rabbit monoclonal Histone H3 antibody from Cell Signaling Technology (PN 4499)
- Alexa Fluor 647-conjugated donkey anti-sheep IgG from ThermoFisher (PN A-21448)
- Alexa Fluor 555-conjugated donkey anti-rabbit IgG from ThermoFisher (PN A-31572)
- Alexa Fluor 488-conjugated donkey anti-mouse IgG from ThermoFisher (PN A-21202)

SINGLE-CELL WESTERN MATERIALS

- Milo scWest Standard Kit from ProteinSimple (PN K600) which includes 8 Standard scWest chips, 10X Suspension Buffer (SB), 5X Wash Buffer (WB), Antibody Diluent 2, Milk-Free Antibody Diluent, and 8 single-use Run/Lysis Buffer vials
- Milo Tweezers from ProteinSimple (PN 035-023)
- Probing Chambers/Sponges Pack of 2 from ProteinSimple (PN A200)

Methods

E18 MOUSE NEURAL TISSUE DISSOCIATION PROTOCOL

The tissue dissociation protocol is described below which has been optimized for isolation of neurons. An overview of the workflow is shown in **Figure 2**.

NOTE: Use tissue immediately after arrival to ensure best results.

1. Pre-warm the NbActiv1 media to 37°C in a water bath.
2. Prepare the cell dissociation solution by adding 3.2 mL He-Ca to a vial of 6.4 mg papain for a final concentration of 2 mg papain/mL He-Ca. Transfer the mix to a 15 mL conical tube and incubate at 37°C for 10 minutes.
3. Using a 1000 µL wide-bore pipette tip, gently transport the tissue and HEB media to a new 15 mL conical tube and let the tissue settle at the bottom.
4. Transfer the HEB medium from the tissue to a new 15 mL conical tube, leaving only enough media to cover the tissue. Keep the HEB media for step 7.
5. Add 2 mL cell dissociation solution to the tissue and incubate at 37°C for 20 minutes, swirling 2-3 times during incubation.
6. Discard the cell dissociation solution without disrupting the tissue at the bottom, leaving only enough solution to cover the tissue.
7. Add ~2 mL HEB media saved from step 4 back to the tissue.

8. Triturate the tissue with the HEB medium 10-15 times or until 90% tissue dispersal is observed using a fire-polished, silanized Pasteur pipette. The tissue should be broken up into small fragments and the media will be cloudy. Allow the tissue debris to settle for 1 minute.

9. Transfer only the supernatant containing dispersed cells to a new 15 mL conical tube and centrifuge cells at 200 RCF for 2 minutes.

10. Discard the supernatant without disturbing the cell pellet.

11. Using a regular-bore pipette tip, resuspend the cell pellet in 1 mL pre-warmed NbActiv1 media.

12. Place a 30 µm cell strainer on top of a new 15 mL conical tube and filter the resuspended cells into the tube.

13. Determine the cell concentration using a hemocytometer. The target concentration is $2x10^6$ cells/mL. To perform a Single-Cell Western experiment on Milo, prepare a 1 mL suspension at 100,000 cells/mL concentration for each scWest chip you plan to run.

SINGLE-CELL WESTERN ON MILO

14. Take 1 mL of the freshly dissociated tissue suspension at 100,000 cells/mL and place onto a rehydrated scWest chip. After 5 minutes, wash away unsettled cells using 1 mL of 1X SB.

15. Run the scWest chip on Milo with the following settings: 10-second lysis time, 85-second electrophoresis time at 240 V, and UV capture for 4 minutes.

16. Probe with rabbit anti-GFAP diluted 1:10 in Antibody Diluent 2 and/or mouse anti-β- III tubulin diluted 1:20 in Antibody Diluent 2 and/or anti-sheep Pax6 diluted 1:10 in Milk-Free Antibody Diluent and/or rabbit anti-Histone H3 diluted 1:40 in Antibody Diluent 2 for 2 hours at room temperature. If probing all four targets together, dilute antibodies in Milk-Free Antibody Diluent.

17. Wash with 1X WB for 3 x 10-minute cycles and then probe with the corresponding Alexa Fluor-labeled secondary antibody/antibodies at a 1:20 dilution in the same antibody diluent as used for the primary antibodies for 1 hour at room temperature, protected from light.

18. Wash the chips with 1X WB for 3 x 15-minute cycles and dry using a 20-second spin in a slide spinner (Labnet) followed by 1-minute air drying with filtered, house air. Image using a Molecular Devices Genepix 4400A microarray scanner. Images were saved as TIFF files and imported into Scout software for quantitative analysis.

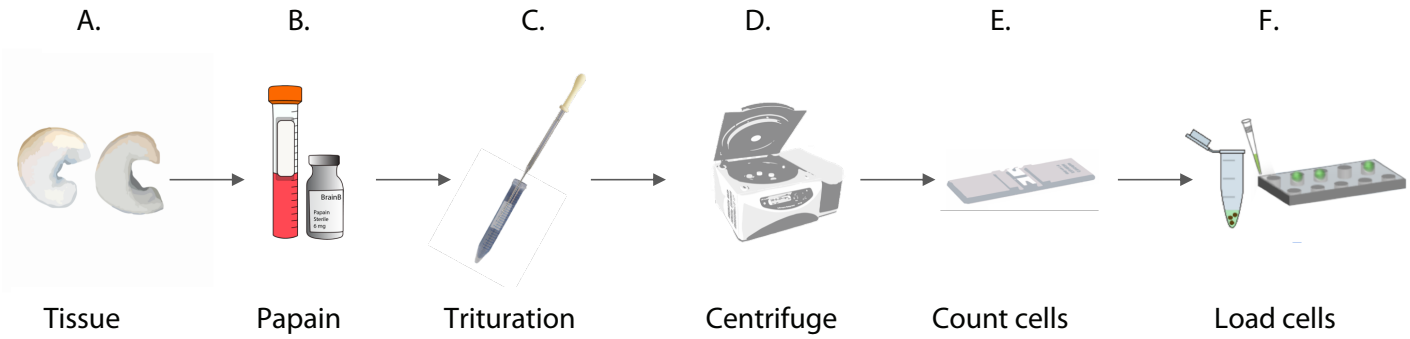

FIGURE 2. Tissue dissociation process, optimized for isolation of neurons. A. Tissue of interest. B. Enzymatic dissociation by papain. C. Mechanical dissociation through trituration in neuronal culture media. D. Spin down cells with centrifugation and resuspend. E. Count cells and dilute to 100K cells/mL in 1X Suspension Buffer. F. Load 1 mL cell suspension on scWest chip and proceed with Single-Cell Western workflow

Results

Single-Cell Western analysis of dissociated neural tissue could clearly detect neuron, astrocyte and radial glia cell types and differentiate between them based on differences in β-III tubulin, GFAP and Pax6 expression, respectively (**Figure 3**). Using Histone H3 as a loading control to identify which lanes on the scWest chip contained single-cells, 735 single-cells were captured and analyzed. All four proteins probed for on the chip gave clean, tight bands after separation and were easily detectable by Scout software.

By using a multiplexed assay design where the same chip was probed simultaneously for Pax6, GFAP, β-III tubulin and Histone H3, we could quantify the number and percentage of cells that were single-target or multi-target positive (**Figure 4**). 17.8% of cells were Pax6+ which is indicative of a radial glia cell phenotype, 1.2% of cells were GFAP+ which is indicative of an astrocyte cell phenotype, and 53.8% of cells were β-III tubulin+ which is indicative of a neuronal cell phenotype. The measured percentage of β-III tubulin+ cells (53.8%) matched the expected neuronal percentage in the tissue (50%) as measured by the manufacturer, highlighting the utility of this protocol specifically for primary neuron analysis. Rarer multi-target positive cell subtypes were also detectable including 1.1% of cells that were Pax6+/GFAP+/Histone H3+ which may suggest some cells exhibit a blended phenotype.

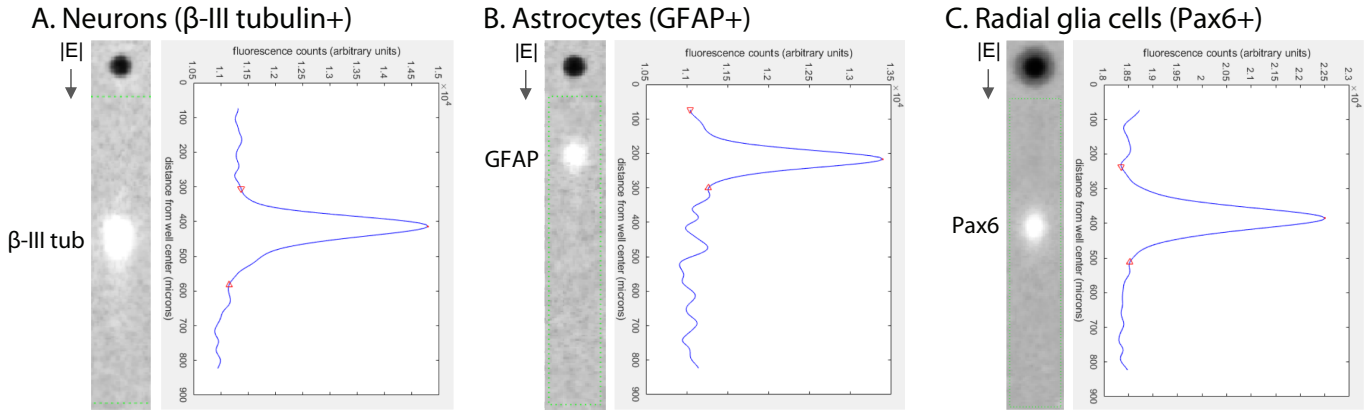

FIGURE 3. Milo identifies (A) neurons (β-III tubulin+), (B) astrocytes (GFAP+), and (C) radial glia (Pax6+) in dissociated neural tissue. Separation images and fluorescence intensity plots show clearly detected protein bands for each target.

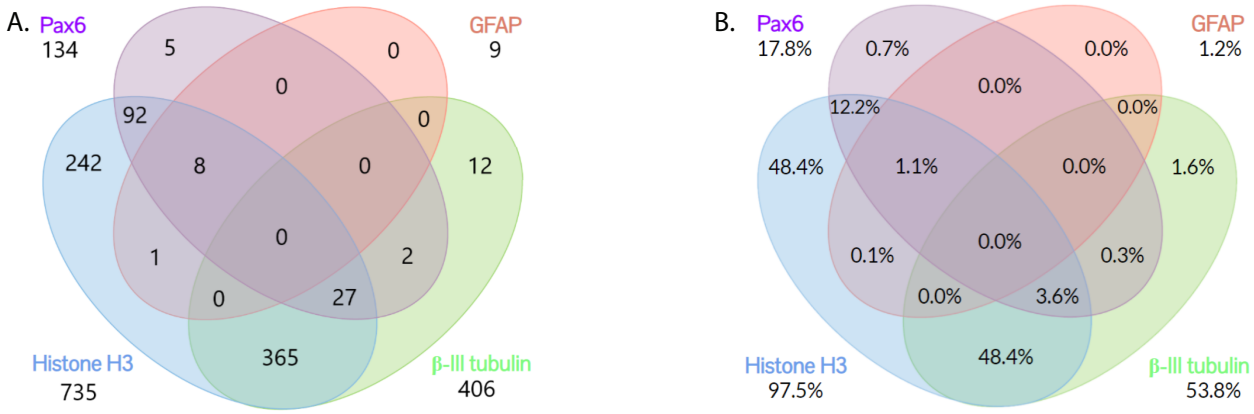

FIGURE 4. Multiplexed 4-target Single-Cell Western assay identifies cell subpopulations in dissociated primary neural tissues. Venn diagrams reveal number (A) and percentages (B) of single- and multi-target positive subpopulations. Measured percentage of β-III tubulin+ cells (53.8%) matches expected neuron percentage of 50% as measured by manufacturer (BrainBits).

Conclusion

Identifying and analyzing the diversity of cell types that are present in the mouse brain is essential to furthering our understanding of both their individual functions and how they interact with each other. Generating high quality single-cell suspensions is an important precursor to a Single-Cell Western experiment in order to preserve cell states and relative abundance in downstream analysis. After generating a single-cell suspension from mouse brain biopsies -- a highly-complex, heterogeneous tissue -- we used Single-Cell Westerns to identify and quantify the abundance of three subtypes of neural cells: neurons, astrocytes and radial glia cells. Using Milo's multiplexing abilities, we quantified individual cells that are multi-positive for a set of neural targets, further stratifying the subpopulations. Future work could examine these subpopulations more deeply by studying expression of different functional or signaling proteins within each cell subtype.

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