Chapter 8

Examination of West Nile Virus Neuroinvasion and Neuropathogenesis in the Central Nervous System of a Murine Model

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Abstract

West Nile virus (WNV) is a neurotropic virus that causes inflammation and neuronal loss in the Central Nervous System leading to encephalitis and death. In this chapter, detailed methods to detect WNV in the murine brain tissue by quantitative real-time polymerase chain reaction and viral plaque assays are described. Determination of WNV neuropathogenesis by Hematoxylin and Eosin staining and immunohistochemical procedures are provided. In addition, TUNEL assays to determine neuronal loss during WNV neuropathogenesis are discussed in detail. Collectively, the methods mentioned in this chapter provide an overview to understand neuroinvasion and neuropathogenesis in a murine model of WNV infection.

Key words West Nile virus, Blood–brain barrier, Neuroinvasion, Neuropathogenesis, Neuronal death

1 Introduction

West Nile virus (WNV) a mosquito-borne, positive-sense, single-stranded RNA virus is a neurotrophic virus with tropism for nervous tissue [1]. WNV invades the Central Nervous System (CNS) to cause inflammation, neuropathogenesis, and neuronal loss leading to encephalitis and death [1–5]. In the United States, WNV is responsible for the increasingly severe encephalitis outbreaks that are reported from horses and humans [2, 5]. Although, WNV is considered as a neurotropic virus, the molecular mechanisms to understand viral entry or neuroinvasion into the brain are poorly understood. The availability of the current murine model serves as a good example to understand the mechanisms of blood–brain barrier (BBB) permeability, neuroinvasion, and neuropathogenesis. The viremia and mechanisms of CNS infections with WNV are not similar in the most common target hosts such as horses and humans [2, 5]. When viremia is high in the blood and other peripheral tissues, WNV has an ability to overcome and cross the extraneural

Tonya M. Colpitts (ed.), *West Nile Virus: Methods and Protocols,* Methods in Molecular Biology, vol. 1435,
DOI 10.1007/978-1-4939-3670-0_8,

and neural barriers by breaching the BBB, the blood–cerebrospinal fluid (CSF) barrier, the CSF–brain barrier, and the blood–spinal cord barrier [1]. Several studies have shown the virological and immunological molecular mechanisms compromise these barriers and allow neuroinvasion of WNV into the brain [6–11]. An overview of the important molecular mechanisms that validate the current mouse model relevant for WNV neuroinvasion needs to be still investigated.

In the murine model, WNV that replicates in the peripheral organs (days 2–4 postinfection p.i.), crosses the barriers (day 5 p.i.) and infects the CNS leading to death of the animals within 2 weeks postinfection [9, 10] (Fig. 1). Detailed in vivo and in vitro BBB models including the Evan's blue staining procedure have been described in the earlier chapter. Also, mice inoculations, perfusions, and isolation of mouse brain procedures have been discussed in the earlier chapter. Here, I have demonstrated the methodology to determine neuroinvasion and neuropathogenesis in murine brains infected with WNV (Fig. 1). This chapter highlights the methodologies for the detection of WNV viral loads by Quantitative real-time PCR (QPCR). Western Blotting using antibodies against the WNV envelope or nonstructural proteins can also be used to detect WNV in the brain. In addition, detection of the higher level of IgG also determines the neuroinvasion of WNV. This chapter does not discuss these other alternative approaches. However, the classical viral plaque assay recommended to determine the virus integrity, viability, and virulence by revealing the virus titers is discussed in detail. The recruitment of immune cells highlighting the process of neuroinflammation and cytokine storms produced by these infiltrated cells suggests the major cause for neuropathogenesis. This chapter discusses the Hematoxylin & Eosin (H&E) staining and immunohistochemical (IHC) methods to determine neuropathogenesis. The neuronal cell death determined by TUNEL assays is also discussed.

2 Materials

Any procedures with WNV-infected mice or brain tissues require the use of a biosafety level III laboratory containment and work practices. Hence it is highly recommended that individuals acquire proper trainings, orientations, and require authorizations to work with WNV. Please follow all relevant regulations and procedures accordingly.

2.1 Neuroinvasion

2.1.1 Mice Inoculation and Brain Collection

1. BD™ U-100 insulin syringe with 27 G×5/8 in. self-contained BD Micro-Fine™ IV permanently attached needle, 1 ml, regular bevel, regular wall. (Orange) (100/sp, 500/ca), Sterile, VWR (Catalog number BD329412).

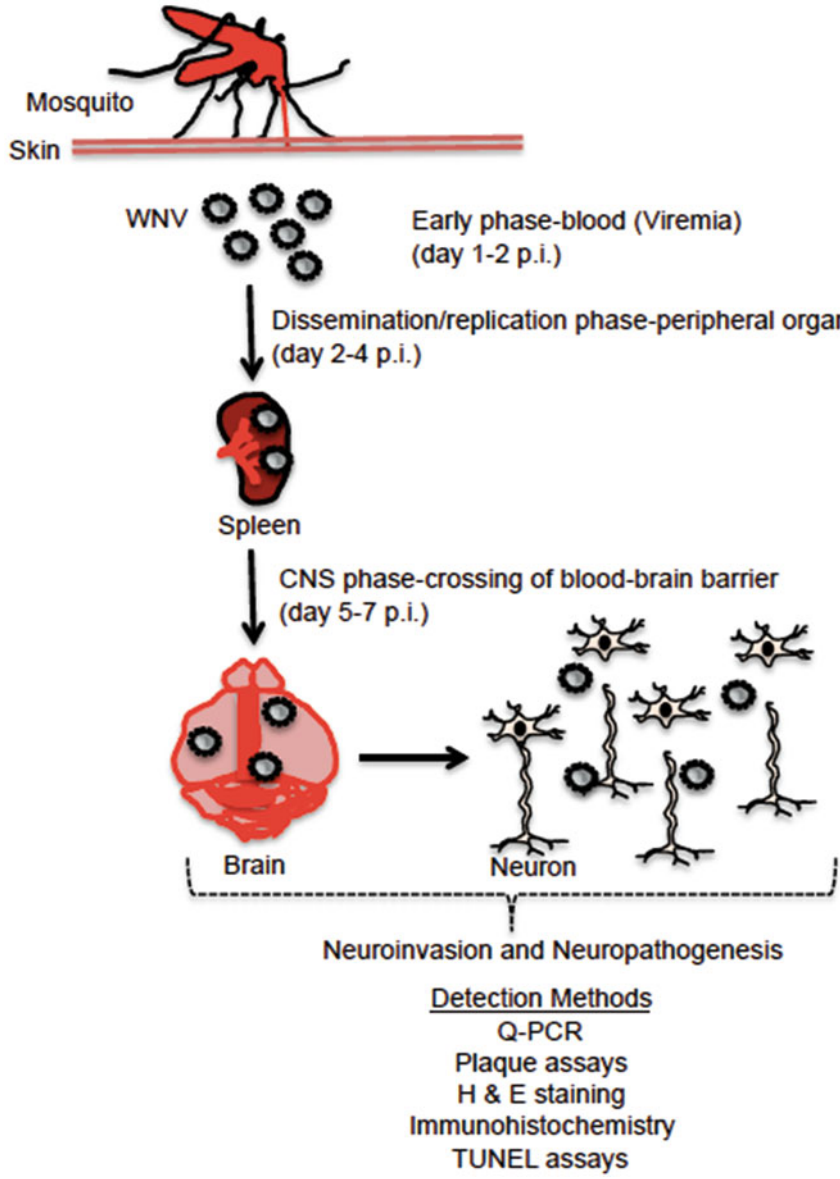

Fig. 1 Schematic representation of WNV pathogenesis and methods used to detect neuroinvasion and neuropathogenesis in a murine model. Overview of the WNV infection kinetics in a murine model and methods used to detect neuroinvasion and neuropathogenesis is shown. In a murine model, high viremia is seen in the blood between day 1 and 2 p.i., followed by dissemination and replication of WNV to peripheral organs such as spleen between days 2 and 4 p.i and infection of CNS between day 5 and 7 p.i. Entry of WNV into the brain causes neuropathogenesis leading to neuronal death. Several methods including but not limited to Q-PCR, H&E staining, immunohistochemistry, and TUNEL assays are used to determine WNV neuroinvasion and neuropathogenesis in the murine brain tissue

2. Phosphate buffered saline (PBS) with 5% Gelatin (Sigma).
3. High-strength autoclavable polypropylene Biohazard bags (VWR).
4. Anesthesia (Forane or Isoflurane), Baxter.
5. Mouse dissection kit with storage case. Includes the following components: Dumont Forcep #5, Vannas scissor, dissecting scissors, wire retractor, needle holder, and blunt probe (Braintree Scientific, Inc; Lab research products) (catalog number MDK-M).
6. Sterile microcentrifuge tubes (any brand).

2.1.2 RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR (QPCR)

1. RNeasy mini kit (50), Includes 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 and 2 ml), RNase-free Reagents, and Buffers (Qiagen; catalog number 74104).
2. RNase-free DNase I (Qiagen) (for on-column DNA digestion).
3. Bench-top Centrifuges (any brand, e.g., Beckman Coulter or Eppendorf).
4. Thermal Cycler (PCR machine) (BioRad or any other).
5. iScript cDNA Synthesis Kit, 100 × 20 µl reactions, includes 5× reverse-transcription reaction mix, iScript reverse transcriptase, nuclease-free water (BioRad: catalog number 1708891).
6. Real-Time PCR detection System (e.g., CFX96 Touch Real-Time PCR Detection System, BioRad or other).
7. Supermixes for Real-Time PCR (e.g., iTaq Universal Supermixes; advanced master mixes that are compatible with any real-time PCR instrument, BioRad).
8. Primers for WNV detection (e.g., forward and reverse primers specific for WNV Envelope or Capsid gene), IDT technologies.
9. Kontes pellet Pestle and microcentrifuge tubes, 1.5 ml, 70 mm length, pack of 100, Kimble-Chase.
10. Kontes Pellet Pestle Grinders, Kimble-Chase.
11. Sterile microcentrifuge tubes (any brand).
12. Brain tissue or lysates in RNA lysis buffer (e.g., RLT buffer from Qiagen kit).

2.1.3 Viral Plaque Assay (Requires a BSL-3 Cell Culture Laboratory)

1. Cell culture growth medium (DMEM high glucose with 4–6 mM glutamine and 10% fetal bovine serum), penicillin and streptomycin (fungizone or antibiotic/antimycotic solutions can be added if required), Life technologies/ThermoFisher Scientific.

2. Ultrapure 2% and 1% SeaPlaque agarose (original low melting temperature agarose ideal for viral plaque assays) (Lonza; catalog number: 50101, 25 g).
3. Plaquing medium (DMEM high glucose with 4–6 mM glutamine and 2% fetal bovine serum and SeaPlaque agarose).
4. 6-well plates (any brand works; e.g. Corning, BD, Co-star, GeneMates).
5. Vero host cells (ATCC).
6. Phosphate buffered saline (PBS).
7. Brains homogenized in PBS.
8. Tissue culture grade sterile water (100 ml).
9. Staining dye for plaque (0.03% Neutral Red) (Sigma).
10. Waterbath (ThermoScientific or any vendor).
11. Standard microwave oven (any brand, domestic).
12. Inverted microscope (any with phase contrast channel).
13. Biosafety cabinet (in a regulated BSL-3 lab) (NuAire or other).
14. CO_2 incubator, set at 37 °C (NuAire or other).
15. Microcentrifuge tubes (any brand).
16. Sterile Pasteur pipettes, serological pipettes, and barrier tips (any brand).
17. Aluminium Foil.
18. Millipore autoclaved water.
19. Sterile glass bottles (Hybex).

2.2 Neuropathogenesis and Neuronal Death

2.2.1 Histology and Immunohistochemistry

1. Phosphate buffered saline (PBS).
2. Fixatives; Preferred standard fixative solution is 10% neutral buffered formalin (100 ml of 37–40% Formaldehyde, 900 ml of distilled Water, 4 g NaH_2PO_4 and 6.5 g of anahydrous Na_2HPO_4); for immunoperoxidase techniques 4% buffered neutral Paraformaldehyde is recommended (8% paraformaldehyde (PFA): 40 g PFA in 500 ml distilled water and 0.2 M Phosphate buffer, pH 7.4 (10.9 g of Na_2HPO_4 and 3.2 g of NaH_2PO_4 in 500 ml distilled water) can be used. While stirring heat 8% PFA solution strictly at 60 °C, once the solution is at 60 °C the PFA is dissolved and 500 ml of 0.2 M phosphate buffer is added to bring the solution to 4% PFA in 0.1 M phosphate. Add two pellets of solid NaOH in 1–2 L of solution or alternatively, add few drops of 1 N NaOH solution, until 4% PFA solution is clear. It is highly recommended to make fresh PFA solution on the same day of fixation, otherwise the 4% PFA can be frozen at –20 °C, but always use as freshly thawed solution. For Bouin fixative Solution add 75 ml of saturated picric acid, 25 ml of 37–40% formaldehyde and 5 ml of glacial acetic acid and mix well (*see* **Note 10**).

3. Mouse brain tissues (frozen or fixed and embedded in paraffin).
4. Histology cassettes (VWR).
5. Hematoxylin Solution (Harris Modified, Sigma).
6. Eosin Y Solution, Alcoholic (Sigma).
7. Resinous mounting medium or Permount histological mounting media (ThermoScientific).
8. Paraffin (ThermoScientific) (*See* **Note 13**).
9. Cryostat containing microtome with sharp knife (Leica or any vendor) (*See* **Notes 13** and **14**).
10. APES (amino-propyl-tri-ethoxy-silane) coated slides (alternative Poly-L-Lysine treatment for slides) (Sigma).
11. Xylene.
12. 100 and 95% Ethanol solutions.
13. Tris/EDTA buffer, pH 9.0 (10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20).
14. Sodium citrate buffer, pH 6.0 (10 mM Sodium Citrate, 0.05% Tween 20).
15. 1 mM EDTA, adjusted to pH 8.0.
16. Antigen retrieval buffer (e.g. Tris/EDTA pH 9.0, Sodium Citrate pH 6.0).
17. Vegetable Steamer or waterbath (set to 100 °C).
18. Container/vessel for slide rack (the vessel should hold roughly 400–500 ml of buffer).
19. Microwave (domestic).
20. Waterbath (set at 37 °C).
21. Slide racks (metal and/or plastic).
22. Humidified chamber (shallow plastic box with a sealed white lid, Nalgene).
23. Wet tissue papers.
24. Wash buffer (TBS and TBS with 0.025% Triton X-100).
25. Block buffer (10% Normal serum with 1% BSA in TBS).
26. Primary antibodies, matching isotype control antibodies and fluorophore-conjugated secondary antibodies and counter nuclear stains (diluted in TBS with 1% BSA) (*see* **Notes 17–19**).
27. Refrigerator.
28. Mounting medium (aqueous-based permanent mounting media).
29. CO_2 incubator (set at 37 °C).
30. Fluorescent or confocal microscope (Olympus or Leica or Zeiss).

2.2.2 Apoptosis (TUNEL) Assay

1. Formaldehyde (4% buffered solution; freshly prepared, prior to use).
2. Phosphate buffered saline (PBS), pH 7.4.
3. Paraffin.
4. Glass slides (pretreated with 0.01% aqueous solution of Poly-L-Lysine from Sigma).
5. Coupling jars.
6. Water or heat baths (set to 60 °C).
7. Xylene bath.
8. Ethanol in coupling jars (96, 90, 80, and 70%).
9. Double distilled or millipore water.
10. Proteinase K stock solution (20 µg/ml; Store for long term at −20 °C) (Sigma).
11. TdT equilibration buffer (2.5 mM Tris–HCl; pH 6.6, 0.2 M potassium cacodylate, 2.5 mM $CoCl_2$, BSA (0.25 mg/ml) (Long-term storage at −20 °C) (*see* **Note 21**).
12. TdT reaction buffer (0.5 U/µl of TdT enzyme and 40 pmol/µl biotinylated-dUTP in TdT equilibration buffer; freshly prepared) (*see* **Note 21**).
13. Humidified chamber (Nalgene; and prepared as described above).
14. Glass coverslips.
15. SSC (2×; 300 mM NaCl, 30 mM sodium citrate, long-term storage at room temperature).
16. BSA solution (2% in PBS) (filter sterilized).
17. TdT staining solution (4× Saline Sodium Citrate Solution; containing 0.6 M NaCl, 60 mM sodium citrate), 2.5 µg/ml fluorescein isothiocyanate-conjugated avidin (Sigma, ThermoScientific), 0.1 %Triton X-100, 1% BSA, prepared freshly prior to use (*see* **Note 21**).
18. Hematoxylin (Sigma) (*see* **Note 11**).
19. Hoechst 33342 (2 µg/ml in PBS; Molecular Probes or Cell Signaling Technologies) (Stored in dark at 4 °C for several weeks) (*see* **Note 22**).
20. Vectashield antifade mounting medium (Vector Laboratories).
21. Fluorescent or confocal microscope (Olympus or Leica or Zeiss).

3 Methods

3.1 Neuroinvasion

3.1.1 Mice Inoculation and Brain Collection

All procedures in this section should be carried out at authorized BSL-3 laboratory and in biosafety containment facilities or cabinets. Researchers should follow all the Biosafety regulations according to their University biosafety office policies. *See* **Note 1**. The previous chapter describes the inoculation of mice and collection of intact brains in detail. Here, I have described these procedures in brief.

1. Age appropriate female C57BL/6 mice (6–8 weeks old) were housed in the ABSL-3 mice facility for 2 days to acclimatize to the new conditions.
2. Biosafety cabinets are divided into two areas as clean or dirty, the diluted virus for inoculation and all supplies in contact with virus should be kept at the dirty side of the cabinet. Supply the biosafety cabinet with container for sharps, biohazard red bags (one bag inside the cabinet and one outside for later use as double bagging) and other required materials.
3. Before inoculation, mice bellies were sterilized with 70% ethanol pads to clear any microbes on the mice or on mice fur.
4. In biosafety cabinets, mice were inoculated intraperitoneal (i.p.) with 1000 Plaque forming units (pfu) of WNV (example: CT2741 or NY99 wild-type parental strains) in PBS with 5% Gelatin, using BD™ U-100 insulin syringe with 27 G×5/8 in. self-contained BD Micro-Fine™ IV permanently attached needle (*See* **Notes 2** and **3**).
5. Needles are immediately disposed in sharps container without capping.
6. Mice were observed for 5–10 min for behavior changes or immediate lethargy or other conditions and cages were decontaminated with 10% bleach and moved to the respective racks.
7. Remaining diluted virus stocks are thoroughly mixed with 10% bleach and discarded along with the original vial (closed) into the Biohazard red bag (inside), double bagged with outside bag and disposed into the regulated waste that gets autoclaved by the ABSL3-regulated facility.
8. Day seven postinfection, mice brains were collected from WNV-infected and control (uninfected) mice by cervical dislocation. Briefly, mice were anesthetized with isoflurane followed by cervical dislocation and brains were isolated using the dissection kit. Brain tissues were collected for RNA isolation in Qiagen RLT buffer (in microcentrifuge tubes) and homogenized (*see* **Notes 4** and **5**) or homogenized in PBS for viral plaque assays (*see* **Notes 6–9**) or otherwise quickly embedded

in histological cassettes and fixed (by completely immersing) in either 10% neutral buffered formalin solution or 4% PFA for immunohistochemistry (*See* **Note 10**).

3.1.2 RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR (QPCR)

Other than proper tissue homogenization (*see* **Notes 4** and **5**), the above procedures are performed with usual general guidelines and as per the manufacturer's instructions.

1. For RNA extraction, WNV-infected and control brain samples (half brain tissue is sufficient for higher RNA yields) were homogenized in 350 µl of RLT buffer containing beta-mercaptaethanol (BME) (according to the manufacturer's instructions). Brains were first homogenized with Kontes Pellet Pestles and Grinders until homogenous solutions has been achieved, further homogenization is performed with passages through 18, followed by 25 G needle and syringes to lyse individual cells (*See* **Notes 4** and **5**). Another 350 µl of RLT buffer with BME is added and the tissue homogenates were kept overnight at −20 °C for lysis to complete.
2. Homogenous brain lysates in RLT buffer were mixed with equal volumes of 70% ethanol and loaded onto RNA columns provided (Qiagen kit). Further steps were performed according to the manufacturer's instructions.
3. The optional step of on-column DNase digestion using DNase I is highly recommended to get pure RNA for sensitive downstream applications such as QPCR. RNA extracted from half of the brain tissue is eluted in 75 µl of RNase-free water. The elution step is repeated with another 50 µl of RNase-free water. Nearly 30–40 µg of RNA is extracted from every 10 mg of homogenized brain tissue.
4. Double-stranded complementary DNA (cDNA) is prepared from 1 µg RNA using iscript cDNA synthesis kit from BioRad (following manufacturer's instructions).
5. Quantitative real-time PCR can be performed with 3–5 µl of cDNA, using BioRad SYBR Green, Primer mix for WNV *Envelope* or *capsid* gene (Forward and Reverse; 1 pmol/µl of each) and nuclease-free water (Sigma).
6. Primers for beta-actin were used in parallel with the primers for Q-RT-PCR normalization. Equal amounts of cDNA samples were used in parallel for beta-actin and WNV *E* gene or capsid gene.
7. Ratio of WNV-*E* or *capsid* gene copy/*beta-actin* gene copy is used as an index to determine the infection rate of each WNV-infected brain samples. QPCR data is representative of results performed in triplicate.

8. Standard curve was prepared using 10-fold serial dilutions starting from 1 ng to 0.00001 ng of known quantities of *WNV-E or beta-actin* fragments as standards.

3.1.3 Viral Plaque Assay (Requires a BSL-3 Cell Culture Laboratory)

1. Vero host cells are plated at a cell density of $1–2 \times 10^6$ per well of a 6-well plate in DMEM high glucose medium with 4–6 mM glutamine and 10% fetal bovine serum, penicillin, and streptomycin. Cells are allowed to grow overnight, in order to reach 65–85% confluency and formation of complete monolayers before infection (*See* **Note 6**).
2. Brain lysate homogenized in PBS is used to determine the WNV titers and virus integrity, viability, and virulence. First six serial dilution of this virus stock is made in labeled sterile microcentrifuge tubes (e.g., add 60 µl of brain homogenate to the first stock tube containing 540 µl of DMEM medium, mix (gently vortex), avoid pipetting as brain homogenates tend to stick to the pipettetip walls. Transfer 60 µl of brain homogenate from first tube to the second tube containing 540 µl of DMEM medium, repeat this to other tubes and make a serial 1:10 dilution of the virus stock.
3. Confluency of Vero host cells is analyzed and DMEM medium is removed using a sterile Pasteur pipette (3 ml). Use of other pipettes may dislodge the cell monolayer (*See* **Note 6**).
4. To each of the dilution well add 500 µl of the $10^{-1}–10^{-6}$ brain homogenate. If plaque assay is performed in duplicate or triplicate, repeat the same for second and third plate.
5. Incubate the plates in a CO_2 incubator, set at 37 °C, for 1–2 h to allow the virus to infect Vero host cells.
6. Microwave or melt 10 ml of sterile (autoclaved) 2% SeaPlaque agarose in water (100 ml glass bottle) and the molten agarose is kept warm at 37 °C waterbath. The culture media is also kept prewarmed to 37 °C before next step (*See* **Notes 7** and **8**).
7. Carefully remove the virus from the host Vero cells using a sterile Pasteur pipette (1 ml) and without dislodging the cell monolayer (*See* **Note 7**).
8. Quickly mix the warm DMEM media and 2% agarose in 1:1 ratio, 1.5 ml of this mix is gently added to make an overlay on the host Vero cells in each well. The plates were incubated for 20 min at room temperature (In BSL3 certified hood) to allow solidification of agarose (*See* **Note 8**).
9. After 20 min, add 1.5 ml of warm DMEM media to each well (Fungizone or antibiotic/antimycotic solutions can be added if required) and transfer the plates carefully to the CO_2 incubator, set at 37 °C (*See* **Note 7**).

10. Incubate plates for 5–6 days, to allow virus replication and infection of the complete monolayer (*See* **Note 9**).
11. After day 6, remove the DMEM media using sterile Pasteur pipettes, add 1 ml of 0.03% Neutral Red in PBS to each well, incubate plates for 2–4 h in a CO2 incubator, set at 37 °C.
12. Remove Neutral Red solution from plates, invert the plates and wrap in Aluminium Foil (dark) and incubate the plates in CO_2 incubator, set at 37 °C, overnight (*See* **Note 9**).
13. Vero cells infected with WNV die and do not take up the Neutral Red stain, however, uninfected cells take up stain and allow to distinguish the plaques as small clear areas against the red and pink backgrounds.
14. Count viral plaques and determine the viral titers on each well: Virus Titer (pfu/ml) = number of plaque × (1 ml/0.1 ml)/fold of dilution.

3.2 Neuropathogenesis and Neuronal Death

3.2.1 H&E Staining

To generate frozen sections, pieces of brain tissue are snap frozen in a cold liquid (liquid Nitrogen) or in cold environments such as −20 or −80 °C freezers. Freezing makes the tissue solid enough to section with a microtome sharp knife. Detailed tissue sectioning has been described in Subheadings 3.2.2 and **Notes 4**. Briefly, frozen sections can be obtained with an instrument Cryostat containing microtome. Make sure the temperature inside the cryostat is about −20 to −30 °C. The tissue sections are collected on a glass slide and processed for immediate staining or embedded in paraffin and kept frozen at −80 °C until use (*See* **Notes 10–15**).

1. For H&E staining, either paraffin or frozen brain sections adhered on slides were first hydrated or deparaffinized. To hydrate the frozen sections (collected freshly) incubate them in three changes of distilled water (3 × 2 min each). If the tissues have to be deparaffinized, sections are incubated for 2 min in each of the following solutions- Xylene (2×), 100% Ethanol (1×), 95% Ethanol (1×), 70% Ethanol (1×) and water (2×). No staining can be performed on tissues containing paraffin, hence the tissues have to be first deparaffinized before processing (*See* **Note 15**).
2. Stain slides with Mayer's Hematoxylin Solution (1 min; if sections were fixed in Bouin's solution, stain for 2 min). Remove excessive water by blotting carefully.
3. Sections are rinsed in warm running tap water for 15 min, avoid the water to run directly on to the slides. Incubate slides in distilled water for 30 s followed by incubation in 95% reagent alcohol for another 30 s.
4. Counterstain the sections with Eosin Y solution (1 min; 2 min for Bouin's fixed sections).

5. Before processing for mounting, sections are dehydrated and cleared in following solutions for 2 min in each; 95% reagent alcohol (2×), 100% absolute reagent alcohol (2×), Xylene (2×). *Dehydration before placing coverslips is discussed in the Notes* (*See* **Note 15**).
6. Mount with resinous mounting medium or Permount histological mounting media.
7. Dry slides overnight and analyze tissues using a microscope.

3.2.2 Immunohistochemistry (IHC)

Tissue samples are too thick for light to penetrate and be transmitted through them. Hence, fixed brain tissues are sliced into very thin sections. Processing of tissues involves several steps: fixation, dehydration, embedment, and subsequent sectioning with a Microtome or a modified microtome, the cryostat device (*See* **Notes 10**, **13–16**). To demonstrate the presence and location of a target protein or antigen in WNV-infected brains, this protocol uses antibodies that recognizes the target protein in fixed tissues [12–15]. Before proceeding with examination and conclusions it is important to optimize a new antibody for antigen retrieval, correct concentrations, and detection. Tips on optimizing a new antibody for IHC are discussed (*See* **Notes 17** and **19**). Although, IHC is less sensitive in comparison to Western Blotting and ELISA, but it provides enormous amount of information on the progression of neuropathogenesis as an intact tissue. In combination with microscopic procedures, IHC on human tissues would provide a complete set of information for pathologist and clinicians. Immunohistochemical staining discussed here uses fluorophore conjugated to the antibody that can be visualized using fluorescence or confocal microscopy.

1. Collect WNV-infected brains and controls in histological cassettes and gently close them. Label the sides of cassettes with pencil and not with pen or permanent markers.
2. Fix the brain tissues in 10% Neutral buffered Formalin solution (preferred fixative over 4% paraformaldehyde) for 24 h. For a successful IHC, proper fixation is highly recommended (*See* **Note 10**).
3. Tissue blocks are embedded in paraffin, and sections are cut to the desired thickness using a microtome. Sections that are five microns are highly recommended for IHC (the thicker ones will raise issues with penetration). Mount the sections on the positively charged amino-propyl-tri-ethoxy-saline coated glass slides or slides treated with Poly-L-Lysine. Dry slides to remove excess water trapped between the section and the slide. Keep slides at room temperature for overnight drying. Alternatively, slides can be kept at 60 °C incubator or oven to allow sections to tightly adhere on to slides (*See* **Notes 13** and **14**).

4. Rehydrate or deparaffinize the slides in a rack by placing them in coupling jars containing series of following solutions; Xylene (2×, 3 min), Xylene 1:1 with 100% ethanol (1×, 3 min), 100% ethanol (2×, 3 min), 95% ethanol (1×, 3 min), 70% ethanol (1×, 3 min), 50% ethanol (1×, 3 min). Run the cold tap water to rinse the slides. Do not dry the slides, but keep them in tap water until ready for antigen retrieval step (*See* **Note 15**).
5. Perform antigen Retrieval by heat mediated (heat-induced epitope retrieval) method, and by using pressure cooker, a microwave, or a vegetable steamer/Rice Cooker/Waterbath set at 100 °C (preferred over others). The enzymatic method is NOT recommended for WNV-infected brain tissue samples, due to higher damage of morphology of the sections (*See* **Notes 16–18**).
6. Set up the Vegetable streamer according to the manufacturer's instructions.
7. Preheat the antigen retrieval buffer (Tris/EDTA pH 9.0, Sodium Citrate pH 6.0) to boil (95–100 °C) in a flask (use domestic microwave as a handy tool to boil the antigen retrieval buffer).
8. Add the hot boiling antigen retrieval buffer to the vegetable steamer container/Vessel and slowly place the slides rack (Metal or plastic) into the vessel, close the vessel lid, and keep the vessel with slides rack inside the steamer and close the steamer lid. Incubate slides in a vessel kept in a steamer for 20 min (*See* **Notes 17–19**).
9. Remove the vessel and run in cold tap water for 10 min (be cautious with the hot solution!).
10. Before immunohistochemical staining, prepare a humidified chamber to avoid the drying of the tissue that may lead to nonspecific binding and high backgrounds. White sealer lid, shallow plastic boxes from Nalgene serves as the best for this purpose. Place wet tissue paper in the bottom and place a square cut parafilm wrap over the tissue paper to avoid the slides touching the wet tissue (*See* **Note 19**).
11. Wash slides (2×, 5 min each) in TBS with 0.025% Triton X-100 with gentle agitation.
12. Block slides in 10% normal serum with 2% BSA in TBS for 2 h at room temperature or overnight at 4 °C.
13. Drain slides and remove excess solution around sections with Kim wipes.
14. Incubate slides with primary antibody (diluted in TBS with 1% BSA), overnight at 4 °C (in humidified chamber to avoid drying) (*See* **Note 19**).
15. Wash slides with TBS containing 0.025% Triton (2×, 5 min).

16. Incubate the sections with fluorophore-conjugated secondary antibody diluted in TBS with 1 % BSA at room temperature for 1 h (in humidified chamber to avoid photobleaching).
17. Rinse with TBS (3×, 5 min), gently overlay coverslips with aqueous-based permanent mounting medium.
18. Dry slides overnight at room temperature and observe under a fluorescent or confocal microscope.

3.2.3 Apoptosis (TUNEL) Assay

Severe neuronal loss and cell death or DNA damage/fragmentation during WNV infection is analyzed by Apoptosis (TUNEL) assays. TUNEL staining is not just restricted for the detection of apoptotic cells, but it can also detect DNA damage associated with nonapoptotic events or undergoing active DNA repair [16, 17]. In situ TUNEL staining for nuclear DNA fragmentation in WNV-infected and control brain tissue sections is described in detail. Sections should be permeabilized with ethanol to allow the TUNEL reaction reagents to penetrate into the cell nucleus. The colorimetric method using Light Microscopy is discussed elsewhere. This chapter describes the TUNEL assay that uses fluorescence staining of tissues (*See* **Notes 20** and **21**).

1. Brain tissues are fixed in 4% Paraformaldehyde (in PBS) for 24 h and embedded in paraffin. Paraffin sections (4–6 μM) were obtained using Cryostat (as described in Subheading 3.2.2) and adhered on to clean glass slides that were pretreated with 0.01 % aqueous solution of Poly-L-Lysine coating material (*See* **Notes 10** and **20**).
2. Sections are deparaffinized by heating the slides for 30 min at 60 °C, followed by incubation in a xylene bath for 5 min at room temperature. All procedures were performed in coupling jars unless otherwise stated (*See* **Note 15**).
3. Rehydrate tissue sections by incubating the slides in series of graded ethanol solutions; 96% ethanol (2×, for 3 min each), 90% ethanol (1×, 3 min), 80% ethanol (1×, 3 min), 70% ethanol (1×, 3 min) and lastly sections were rehydrated with double distilled water (1×, 3 min). Excessive water is carefully removed by blotting.
4. Incubate slides with 20 μg/ml of Proteinase K solution for 15 min at room temperature, then wash slides with double distilled water (3×, 5 min each). Excessive water is carefully removed by blotting.
5. Incubate slides with TdT equilibration buffer for 10 min at room temperature, and then remove buffer. *Lower volumes of TdT buffers can be used if desired* (*See* **Note 21**).
6. Carefully cover sections with TdT reaction buffer, incubate slides in a prepared humidified chamber for 30 min at 37 °C.

7. TdT reaction is stopped by incubating the slides in 2× SSC (2×, 10 min each), slides were rinsed in 1× PBS.
8. Block the nonspecific binding by incubating slides with 2% BSA solution for 60 min at room temperature or overnight at 4 °C.
9. Wash slides with 1× PBS (2×, 5 min each), cover the sections in TdT Staining buffer, and incubate at room temperature for 30 min in dark humidified chamber. Wash Slides again with 1× PBS (2×, 5 min each) (*See* **Note 21**).
10. Stain sections with hematoxylin, Hoechst 33342, or other appropriate counterstains. *See* **Note 22** *on the benefits of Hoechst counterstaining.*
11. Wash slides with 1× PBS and gently adhere coverslip using Vectashield antifade mounting medium. Air dry slides (room temperature) in dark humidified container for overnight.
12. Keep slides at 4 °C, until ready for use. Examine the brain tissue sections using fluorescent or confocal Microscope.

4 Notes

1. As mentioned before, all procedures with WNV-infected brain tissues should be carried out at an authorized and regulated BSL-3 or ABSL-3 laboratory and in Biosafety cabinets designated separately for cell culture or animal procedures.
2. It is required to determine the virus titers and also test different mice backgrounds and different WNV strains for neuroinvasion and neuropathogenesis.
3. *Mice/brain tissues*: Researchers can anesthetize mice with recommended amounts of Isoflurane before inoculation of WNV, although, it is preferred to inoculate mice without anesthesia for quick recovery after infection.
4. All virus dilutions are done in a BSL-3 cell culture laboratory and the diluted virus stocks are carried to the ABSL-3 facility in a safe double container. WNV-infected brain samples can be transferred to the BSL-2 laboratory in RLT buffer that contains high concentration of guanidine isothiocyanate, which completely inactivates the virus.
5. For complete homogenization of brain lysates, it is strongly recommended to lyse brains by both Pestle-grinder and needle-syringe passages. Extreme care should be taken during the needle-syringe passages. After use, throw the needles in sharps containers without capping.
6. *Viral plaque assays*: Vero cells are recommended. But upon unavailability baby hamster kidney (BHK) cells can be used.

Do not overgrow host cells above 85% confluency, as cells overgrown tend to dislodge monolayers easily. Also, it is recommended not to swirl the cells as this would lead to lower cell numbers in the plate center. For use on same day it is suggested to plate $2–3 \times 10^6$ cells per well, to allow the formation of a complete monolayer.

7. Fungizone or antibiotic/antimycotic solutions can be added if required, as plaque assay plates usually gets fungus growth within 5–6 days of incubation.
8. Keep the culture media and agarose at 37 °C waterbath and check the appropriate temperature with a thermometer, slight increase in temperature would dislodge the Vero host cell monolayer. Culture media is also prewarmed to 37 °C, in order to keep agarose in molten state and not to solidify.
9. It is not advisable to keep plaque assay plates for more than 4 h in staining with Neutral Red, as living cells would take up more dye and give hard time to distinguish the lighter areas. Also, using more Neutral Red would result in clear identification of plaques. If desired, plaque assay plates can be kept for 5–8 days (if fungizone is added to avoid fungus growth).
10. *H&E staining*: Use the "Progressive" Hematoxylin stain that gives a desired intensity of staining for frozen sections when dipped in the stain solution. Try to avoid over staining with Eosin. Also avoid excessive water carry over by timely blotting the sections.
11. If tissues are kept in reusable cassettes, make sure that there are no carried-over tissues to avoid "floaters" problems that may occur during and after staining of sections. It is very important to identify any remaining tissue and wash the cassettes thoroughly with running tap water.
12. *Fixation*: Immediately after removing the brain tissue, keep in histological cassettes and completely immerse in the fixative solution to preserve the tissue. The volume of fixative should be 10:1 ratio of fixative to tissue. Although, 10% neutral buffered formalin solution is relatively slow, but it penetrates the tissue well and prevents acidity that would promote formation of formalin-heme pigments that appear as black-polarized structures/deposits in tissues and upon tissue damage and autolysis. Also, fixation is carried out best at neutral pH, in the range of 6–8. Commercial formalin solutions are preferred as their are buffered at a pH of 7 with phosphate. The 4% paraformaldehyde is good for immunoperoxidase staining. To avoid the exhaustion of the fixative, it can be changed at intervals. Avoid too high concentrations of fixatives and drying of tissues (always keep tissues moist with PBS) that may give adverse effects and artifacts (cellular organelle loss, nuclear shrinkage, and clumping).

13. *Sectioning*: Using a sharp knife microtome, cut thin microscopic sections (2–3 mm), to allow best penetration of fixative solution into thin sections. Paraffin is highly recommended for tissue embedding in comparison to other alternatives, such as less expensive plastics (that may allow much thinner sections). But, plastic requires special reagents for dehydration and clearing and a special microtome with a glass or diamond knife. Tissues embedded in paraffin will result in 8–10 thin sections. Before embedding or infiltration in to paraffin, tissues are dehydrated in a series of alcohols. After dehydration, use less expensive Xylene as the best clearing agent to remove the dehydrants. Depending on the climate and season, paraffin can be obtained with different melting points.
14. Sectioning needs time, patience, and real skills that comes over with practice. When sections are done carelessly, several artifacts such as folding, ripping, holes, and blinds are generated. To help remove the folds and wrinkles, sections are floated in warm waterbaths and picked on microscopic glass slides. Slides are incubated in warm oven for 15 min to allow adherence of the sections. If heat can harm the antigens for immunostaining, use glue-coated slides to pick up the sections and skip the oven incubation step.
15. Before staining, *deparaffinize* or dehydrate the sections by running through the recommended solution of Xylene, alcohols, and water. If paraffin is not completely removed, staining will not work on tissues containing paraffin.
16. *Antigen retrieval*: During tissue fixation, methylene bridges are formed, that may cross-link the proteins and hide/mask the antigenic sites. To expose the antigen binding sites, it is highly recommended to perform antigen retrieval procedures.
17. Optimize the new antibodies for *antigen retrieval*, concentration/dilution, and detection, as each new antibody will have its preferred method. Detailed protocol for heat-induced antigen retrieval using vegetable steamer is provided in this chapter, however, there are other methods (described elsewhere) to perform antigen retrieval using a pressure cooker or microwave or waterbath set at 60 °C to incubate slides for overnight in antigen retrieval solution. If the antibody datasheet does not describe the method for antigen retrieval, it is advised to test for antigen retrieval using different methods. Also, the enzymatic methods using horseradish peroxidase (HRP) or alkaline phosphatase (AP) and chromogens for immunoperoxidase staining are described elsewhere.
18. For heat-induced antigen retrieval using vegetable steamer, 20 min of retrieval time is suggested. However, it is recommended to perform a test experiment with 5, 10, 15, 20, 25,

and 30 min to find out the correct retrieval time for any particular or new antibody. Less than 20 min may result in under-retrieval of antigens and weak immunostaining and over 20 min may cause over-retrieval, nonspecific background staining and damage of the tissue sections and disassociation from the slides. Also, it is highly recommended to cool the slides after 20 min of incubation, for easy handling and allowing the antigenic sites to re-form after being heated at high temperatures.

19. *Optimization of antibodies*: Use appropriate antigen retrieval method and correct optimized amounts of the primary and secondary antibodies for detection. Dilutions of the antibodies are sometimes provided on the datasheets (by the manufacturer), otherwise determined by the end-user by testing a range. Most of the primary antibodies work great between 0.5 and 10 μg/ml concentrations. It is highly recommended to check that the primary antibody is not raised in a mouse species similar to the tissue sections being stained, otherwise anti-mouse IgG secondary antibody would bind to all the endogenous IgG in the mouse tissue, leading to high nonspecific staining. In case of lower antibody titer or targets with less affinity, longer incubation times with the primary antibody is recommended to allow sufficient time for binding. Using Isotype control antibodies directed to an irrelevant target or antigen or negative or positive control antibodies are highly recommended to ensure the immunostaining of the experimental antibodies. Also, a control tissue with no expression of target protein can be used as a negative tissue control.
20. *TUNEL assay*: Optimization of formaldehyde fixation is required, it can be resolved with shorter fixations or reducing the concentration of formaldehyde.
21. False positive staining in the negative controls may arise due to nonspecific binding of the FITC-conjugated reagent used for fluorescence detection. Reduce the fluorophores and incubation times to avoid the background fluorescence. Optimize the staining conditions for controls prior to experimental set up. The control panels are highly recommended in TUNEL staining to help interpret the data. Positive controls can be treated with DNAse I (1 μg/ml in 30 mM Tris–HCL (pH 7.2), 140 mM Potassium cacodylate, 4 mM $MgCl_2$, 0.1 mM DTT) for 10 min at room temperature. Wash samples for 3× (2 min each) with double distilled water and then proceed with TUNEL. Omitting TdT enzyme from TdT reaction can serve as negative control. If lower volumes of TdT buffers are preferred, carefully cover the buffers using a glass coverslip. Care should be taken to avoid trapping air bubbles that may lead to staining and fluorescence artifacts.

22. Hoechst 33342 staining enables comparison of TUNEL positive nuclei and changes in nuclear size and morphology. Also, it can serve as counter stain for the visualization of gross anatomical changes in TUNEL positive and negative tissues.

Acknowledgement

This work is supported by start-up funds from Old Dominion University to H.S. The author would like to acknowledge Dr. Girish Neelakanta for critically reading and commenting on this chapter.

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