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Analysis of HSV viral reactivation in explants of sensory neurons

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Abstract

As with all Herpesviruses, Herpes simplex virus (HSV) has both a lytic replication phase and a latency-reactivation cycle. During lytic replication, there is an ordered cascade of viral gene expression that leads to the synthesis of infectious viral progeny. In contrast, latency is characterized by the lack of significant lytic gene expression and the absence of infectious virus. Reactivation from latency is characterized by the re-entry of the virus into the lytic replication cycle and the production of recurrent disease. This unit describes the establishment of the mouse sensory neuron model of HSV-1 latency-reactivation as a useful *in vivo* system for the analysis of mechanisms involved in latency and reactivation. Assays including the determination of viral yields, immunohistochemical/immunofluorescent detection of viral antigens, and mRNA quantitation are used in experiments designed to investigate the network of cellular and viral proteins regulating HSV-1 lytic infection, latency, and reactivation.

Keywords

Herpes Simplex Virus; latency model; corneal infection; trigeminal ganglion; small molecule inhibitors; viral titer; immunohistochemistry

Herpesviridae comprises a family of double stranded DNA viruses with biphasic replication cycles characterized by stages of productive lytic infection and recurrent latency-reactivation cycles (Roizman et al., 2007). During lytic replication, there is an ordered cascade of viral gene expression that leads to the production of infectious progeny. In contrast, latency is characterized by the repression of lytic viral gene expression and the absence of infectious virus. Herpes simplex virus 1 (HSV-1) primary lytic infection results in the productive replication of the virus at the initial site of infection, typically in epithelial and fibroblast cells. Progeny virus may then infect sensory neurons at axonal terminals located near the primary site of infection. The virus travels along the axon through retrograde transport to the nucleus. Within the neuronal nucleus, the viral genome circularizes and is assembled into complex nucleosome structures that block lytic viral gene expression and promote the expression of the viral non-coding Latency Associated

Transcripts (LATs) and a select set of microRNAs. This pool of latent genomes thus represents a viral reservoir that persists for the life of the host.

Latent HSV-1 can reactivate to produce infectious virus, although the molecular signals and mechanisms remain unclear. Cycles of reactivation result in the production of recurrent disease that can range from mild herpetic lesions to life threatening encephalitis (Whitley et al., 2007). Additionally, congenital infections can lead to persistent neurological issues. Importantly, HSV herpetic keratitis is also the foremost cause of viral-mediated blindness in the developed world. Thus, there remains an important need for models to investigate the molecular mechanisms of HSV-1 latency-reactivation cycles as well as a clinical need for the development of effective antivirals.

This unit describes protocols to establish a mouse model for the investigation of HSV-1 latency and reactivation. Methods detailed include animal handling procedures and ocular infection of Balb/c mice (see Basic Protocol 1) to establish latency in the sensory neurons of the trigeminal ganglion. Following the establishment of latency, trigeminal ganglia can be harvested (see Basic Protocol 2) and induced to model viral reactivation through *ex vivo* explant (see Basic Protocol 3). Additionally, this model is applicable to the investigation of small molecule inhibitors targeting cellular or viral factors to determine specific pathways and components involved in the HSV-1 latency reactivation cycle. Detailed methods are provided for generating viral stocks (see Support Protocol 1), determining viral titers (see Basic Protocol 4), as well as immunohistochemical/immunofluorescent staining (see Basic Protocol 5) of explanted trigeminal ganglia.

CAUTION: Human herpes simplex virus is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See **UNIT 1A.1** (Burnett et al., 2009) and other pertinent resources (**APPENDIX 1B: Resources for International Biosafety Guidelines and Regulations**) for more information.

CAUTION: These experiments require Animal Biosafety Level 2 (ABSL-2) conditions. Follow all appropriate guidelines for the use and handling of infected animals. See **UNIT 1A.1** (Burnett et al. 2009) and other pertinent resources (**APPENDIX 1B: Resources for International Biosafety Guidelines and Regulations**) for more information. Protocols using live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to NIH regulations regarding the care and use of laboratory animals.

NOTE: All solutions and equipment coming into contact with animals and living cells must be sterile. Aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified, 37°C, 5% CO₂ incubator unless otherwise specified.

BASIC PROTOCOL 1

ESTABLISHMENT OF HSV-1 LATENCY IN THE MOUSE MODEL BY CORNEAL INFECTION

While HSV is primarily a human pathogen, inbred and transgenic mouse strains have been widely utilized in the study of latency, reactivation, and recurrent disease. This section describes the establishment of the HSV-1 ocular latency model.

For efficient infection, the corneas of anesthetized Balb/c mice are first scarified prior to addition of HSV-1 viral inoculum to the surface of the eye. From the site of infection, HSV-1 infects sensory neurons at axonal terminals and establishes latency in the neurons of the trigeminal and other sensory ganglia.

Materials—Animal Biosafety Level 2 (ABSL-2) conditions

Animal cages

Absorbent paper

Balb/c mice, 5 to 6 weeks old, acclimated for 1 week

1cc syringes with 26 to 27-gauge needle

Avertin, 20 mg/mL (see recipe)

Syringes with 30-gauge needle

Ice bucket or cooler

HSV-1 strain F viral stock diluted in DMEM/FBS 1% (see Support Protocol 1 for more information)

Pipetman and plugged tips

Heat lamp

Preparation of procedure and animal handling—

1. Acclimate 5–6 week old Balb/c mice for 1 week at the animal facility prior to infection.

Acclimating the mice prior to infection reduces the level of stress for the animal and thus decreases the likelihood of stress-induced “enhanced infection”.

2. Prepare for the infection in a laminar flow biological safety cabinet (BSL-2):
 - a. Fill 1cc syringes equipped with 26 – 27 gauge needles with Avertin.
 - b. Place appropriately diluted HSV-1 viral stock on ice or in a cooler.
3. Combine two cages of mice (total 10), maintaining the empty cage for anesthetized animals. Remove one animal by the tail. With the opposite hand,

gently but firmly grab the skin at the back of the neck with thumb and forefinger (see Figure 1). Pull gently on the tail to stretch the animal and secure the tail with pinkie finger. At this point the mouse should be firmly restrained in one hand.

Sedation of mice—

4. With the opposite hand, hold a syringe filled with Avertin and position the body of the mouse such that the abdomen is above the head. Carefully insert the needle at a 30-degree angle into the abdomen of the mouse just left of the midline, but above the nipple. Ensure the needle has entered a depth of 0.5 – 1 cm, and slowly inject 0.25–0.3 mL into the abdomen of the mouse. Return the mouse to the empty cage and cover.

NOTE: Positioning the abdomen of the mouse above the head during intraperitoneal injections reduces the likelihood of puncturing the bladder or intestines.

NOTE: Avertin is the anesthetic of choice to maintain the animal immobile for ~ 1 hr to allow for efficient infection. However, inappropriate dosage of Avertin can be lethal to the animal. Each lot of prepared Avertin must be carefully tested to empirically determine the appropriate dose. Avertin should always be made from freshly obtained stock solutions.

NOTE: Following intraperitoneal injection of Avertin, the mouse may exhibit a short period of hyperactivity (~5 minutes) prior to sedation. Once sedated, the mouse should remain in this state for approximately 1 hour.

5. Repeat steps 3 – 4. In general it is convenient to anesthetize 10 mice prior to proceeding to the next stage (ocular scarification/Infection).

Ocular scarification—

6. Once sedated, remove the mouse from cage and place on the surface of the BSL-2 cabinet with the abdomen facing down and the head turned to one side.
7. With your thumb and index finger, gently press down on the skin surrounding the eye until the eye protrudes from the socket (Figures 2a and 2b).
8. With a 30-gauge needle in your other hand, stroke 4 – 5 times across the eye making sure that the bevel of the needle is facing up (Figure 2c). Repeat procedure on the second eye. Continue with the next animal until 5 (one cage) have been scarified.

You should experience a slight resistance when scarifying the eye. However extreme care should be taken to avoid puncturing the eye.

Replace the needle after the scarification of 10 animals.

Scarification of the eye enhances HSV-1 entry. Care should be taken when performing scarifications since the efficiency and reproducibility of infection between animals is highly dependent on the level of scarification. Scarification by different investigators can result in different levels of infection.

Ocular infection—

9. Return animals to the empty cage with abdomens facing down and the heads positioned forward.
10. With your thumb and index finger, gently move the skin away from the area around the eye.

In contrast to scarification, here the objective is to simply expose the surface of the eye for infection.

11. Infect (see note for strain dependence) by pipetting 2 μ l of the viral stock (i.e. HSV-1, Strain F, 5×10^5 pfu/ μ l) directly onto the surface of the eye. Repeat the procedure for the second eye. A small bubble of liquid will remain on the eyes.

HSV-1 strains F and KOS are less virulent than strains McKrae and 17syn+. For strains McKrae and 17syn+, the infectious dose is generally less than F or KOS. However, McKrae and 17syn+ are reported to have higher levels of spontaneous reactivation, which can be advantageous depending upon the goals of the experiment (Webre et al., 2012).

12. Return the cage to the storage area taking care not to disturb the bubble from the eye.
13. Animals should recover from Avertin within 1 hr. If animals have difficulties recovering and/or labored breathing, warm the animal in your cupped hand or place on a towel under a heat lamp (low temperature setting) to prevent hypothermia.
14. Animals are left to undergo primary infection and progress to the establishment of latency in the sensory ganglia. Evidence of primary infection (lesions on and around eyes with some hair loss) will be evident between 5–12 days post infection. During this period, a percentage of animals may succumb to encephalitic infection (evident by partial paralysis and/or labored breathing). As noted, the percentage is dependent upon the viral strain and the initial infection dose. In some cases, these animals will ultimately recover. However, the disposition of these animals will be specified in each individual investigator's approved animal protocol. By day 14 post-infection, animals will exhibit signs of healing and resolution of infection. The surviving animals are held for 4–8 weeks post infection to allow for the full establishment of latency.

SUPPORT PROTOCOL 1

PREPARING VIRUS STOCKS

Methods utilized for the generation of viral stocks through the infection of tissue culture cells are widely described in the literature [(Brown and MacLean, 1998) and **UNIT 14E.1.1** (Blaho et al., 2005)]. This section describes the preparation of a large stock of HSV-1 strain F that is adaptable for the use in the infection of both animal models (see Basic Protocol 1) and tissue culture cells [see **UNIT 14E.5** (Turner et al., 2014)]. In addition, this protocol has been optimized to reduce the amount of defective viral particles and recommends storage of viral inoculum in multiple aliquots to eliminate freeze-defrost cycles.

Materials—Vero cells (ATCC, Cat.# CCL-81)

PBS

0.25% Trypsin-EDTA

DMEM/5% FBS medium (see recipe)

150×25 mm tissue culture dish(s)

Hemocytometer

HSV strain F stock

DMEM/1% FBS medium (see recipe)

Cell scraper (Sarstedt, Cat.# 83.1830)

50 mL conical centrifuge tube

Sonicator equipped with micro-tip probe

Sterile 1.5 mL tubes

Plating and infection of Vero cells—

1. Maintain a culture of Vero cells in DMEM/5% FBS. See **APPENDIX 4E** (Ammerman et al., 2008) for information regarding the growth and maintenance of Vero cell lines.
2. Plate sufficient numbers of 150×25 mm plates containing Vero cells to generate viral stocks.
On the day of infection, ensure 150×25 mm dishes are 90–100% confluent monolayer.
Typically 15–20 dishes are sufficient for preparing virus stock.
3. Count cells using a hemocytometer or automated cell counter. Determine the total number of cells per dish.

4. Based upon cell count, dilute HSV-1 strain F stock to 0.1 PFU/cell in DMEM/1% FBS medium.

$$(\# \text{ of cells})(\text{desired PFU/cell}) = \text{total PFU needed}$$
5. Aspirate media from cells and transfer viral inoculum to dishes. Transfer dishes to a 32°C/5% CO₂ incubator equipped with a rocking platform. Rock cells at 10 rpm for 1 hr to allow absorption of the virus into the cells.
Alternatively, if a rocker is not available during the viral adsorption stage, plates can be gently rocked every 20 min by hand to ensure equal distribution of the viral inoculum across the monolayer.
6. Following the 1 hr absorption, aspirate viral inoculum and replace with DMEM/5% FBS medium. Transfer plates to 32°C/5% CO₂ and incubate for 3 – 5 days.
Cytopathic effect (CPE) should become apparent between 1 – 2 days post-infection. The cells should be ready to harvest between 3 – 5 days. At this stage, nearly all of the cells should display significant amount of CPE (cells rounding and detaching from plate).
Allowing the infection to proceed at 32°C and not 37°C reduces the amount of defective viral particles.

Harvesting cell associated virus—

7. Scrape infected cells into medium and transfer to a 50 mL conical centrifuge tube. Collect infected cells by centrifuging for 5 min at 1000 rpm, 4°C.
8. Aspirate media and freeze pellet on dry ice. For long-term storage, place at –80°C.
The initial freezing of infected cell pellets aids in disruption of the cellular membranes.
9. Defrost infected cell pellets on ice and resuspend with DMEM/1% FBS medium.
 Generally 1 – 2 mL DMEM/1% FBS medium per 10 dishes.
10. Using a sonicator equipped with a micro-tip probe, sonicate each sample for 20 – 60 sec (on ice) or until the cell pellet has been satisfactory disrupted.
Sonication conditions are dependent on the make and model of the sonicator. Optimal conditions for Branson Sonifer 450D are 10% amplitude, 2 – 3 cycle, 20 sec.
During sonication the probe may generate significant amount of heat. Sonication steps should be completed at 4°C with the tube placed in an ice water bath.
11. Clarify the sonicated lysate by centrifuging for 10 min at 1000 rpm, 4°C. Transfer the supernatant to a sterile tube.

If a relatively large pellet remains, optimize sonication conditions in step #10.

12. Aliquot desired volume of infected cell lysate into sterile microfuge tube(s) and freeze immediately on dry ice. For long-term storage, place at -80°C .

Aliquot infected lysate into multiple tubes (25 – 50 μl per tube) to eliminate freeze-defrost cycles.

13. Proceed to viral titering (Basic Protocol 4).

BASIC PROTOCOL 2

ISOLATION OF TRIGEMINAL GANGLIA FROM THE HSV-1 LATENCY MOUSE MODEL

Following corneal infection of mice with HSV-1, the virus enters sensory neurons at axonal terminals. Cellular as well as viral factors that regulate the dynamics of HSV-1 latency and viral reactivation cycles can be characterized using the trigeminal ganglion of latently infected mice (see Basic Protocol 1).

The isolation of the trigeminal ganglion from latently infected mice involves a degree of skill to remove the delicate tissue. It should be noted that death/injury is an effective stimulus to reactivate latent HSV-1. As such, the trigeminal ganglion should be recovered in as rapidly a timeframe as possible if the desired studies will examine viral latency or initiation stages of viral reactivation. Alternately, *ex vivo* explant of the trigeminal ganglion can be utilized to investigate later time points in the reactivation process (see Basic Protocol 3).

Materials—Animal Biosafety Level 2 (ABSL-2) conditions

HSV-1 latently infected Balb/c mice (see Basic Protocol 1)

Absorbent paper

Dissecting board

Surgical pins

Iris/curved scissors (Vantage, V95–306, 10.5cm)

Half curved eye dressing forceps (Vantage, V918-782, 10.2cm)

Curved-sharp tweezers (Sigma-Aldrich, T4787, Style #7)

Micro-dissecting spring scissors (Roboz, RS-5658, 3.5")

Tissue culture dish

Phosphate-buffered saline (PBS)

Liquid nitrogen

Mouse dissection—

1. Place dissecting board covered with absorbent paper, instruments, and HSV-1 latently infected mice into laminar flow biological safety cabinet (BSL-2).
2. Euthanize HSV-1 latently infected mice according to institutional (IACUC) guidelines. Note that rapid methods of euthanasia are preferred to minimize the time required to remove the trigeminal ganglia.
3. Place the mouse abdomen side down on a dissecting board covered with absorbent paper.
4. Immobilize the euthanized mouse onto the dissecting board by inserting a surgical pin through the nasal cavity (Figure 3b). Stretch the animal by pulling gently on tail and inserting a second surgical pin through the hindquarters, just above the base of the tail.
5. Expose the top of the skull by removing the skin, including ears, with iris/curved-scissors (Figure 3c).
6. Insert the tip of curved forceps into the base of the skull, near the spine. Gently lift skull and insert iris/curved-scissors. Cut upwards along the circumference of the skull without disturbing the brain (Figure 3d).

With HSV-1 ocular infections, regions of the mouse brain have been shown to be potential reservoirs of latent virus and could possibly be used in further experiments (Burgos et al., 2002; Vann and Atherton, 1991).

7. Once the skull has been cut through, carefully lift the top of the skull to expose the brain (Figure 4a).
8. Using the backside of curved forceps, remove the brain in a “scooping” motion without disturbing the paired trigeminal ganglia located directly below the brain at the base of the skull.

Removal of trigeminal ganglion—

9. The anterior section of trigeminal ganglion passes below the bridge of the eye socket. Gently remove the bridge of the eye socket using iris/curved-scissors and curved forceps (Figures 4b and 4c).

The trigeminal ganglion is extremely fragile and extra care should be taken if the tissue will be stained via immunohistochemistry (Basic Protocol 5).

10. With curved-sharp tweezers, begin to lift the trigeminal ganglion at the rear of the skull and snip carefully underneath to release the ganglion using micro-dissecting spring scissors (Figure 4e).
11. Once the trigeminal ganglion is removed (Figure 4f), place in a tissue culture dish with PBS.

12. Repeat steps 9–10 to remove the second trigeminal ganglion.
13. At this point, the ganglia can be explanted into tissue culture media for reactivation studies (continue to Basic Protocol 3, 4, or 5), processed for viral DNA analyses [see **UNIT 14E.5** (Turner et al., 2014)], or flash frozen in liquid nitrogen. To flash freeze, place ganglia in a cryotube and immediately incubate in liquid nitrogen for ~10 min. Frozen ganglia may then be transferred to a liquid nitrogen freezer for long-term storage.

BASIC PROTOCOL 3

MOUSE GANGLIA EXPLANT-REACTIVATION MODEL

Within the natural host (humans), HSV-1 establishes latency within the sensory neuron. Various stress stimuli (tissue injury, ultraviolet light, heat shock, hormonal alterations) or immunosuppression can induce reactivation. This shift in the dynamic from latent to lytic infection can lead to viral shedding as well as the development of herpetic lesions at the initial site of infection.

In the mouse model described, reactivation *in vivo* has been demonstrated by various stimuli including exposure of the infected mouse to hyperthermal stress, UV irradiation, or glucocorticoid treatment (Cook et al., 1991; Sawtell and Thompson, 1992; Shimeld et al., 1990). However, these methods are less efficient at stimulation of viral reactivation than the direct isolation and explant of the trigeminal ganglion into culture (Liang et al., 2013b; Liang et al., 2009) as described in this protocol. Furthermore, the use of small molecule inhibitors specifically targeting cellular or viral factors can be added to the explant culture to determine whether specific proteins are involved in the HSV-1 latency-reactivation cycle.

Materials—6-well tissue culture dishes

DMEM/5–10% FBS medium (see recipe)

Optional: Small molecule inhibitors, inhibitory or stimulatory compounds, or antibodies

Curved-sharp tweezers (Sigma-Aldrich, T4787, Style #7)

Set of two surgical scalpels (Bard-Parker, 1310, size 10)

HSV-1 latently infected mouse trigeminal ganglia

Liquid nitrogen

1. Working in a laminar flow biological safety cabinet (BSL-2), add DMEM/10% FBS medium into the appropriate number of wells.

Transfer 2 mL of DMEM/10% FBS medium per well of 6 well tissue culture dish for standard explant of ~5 ganglia per well or 0.5 mL per well of a 24 well dish for 0.5–1 ganglia per well.

2. Using curved-sharp tweezers, transfer the trigeminal ganglion (see Basic Protocol 2 for dissection) into the 6-well dish containing DMEM/10% FBS medium.

Small molecule inhibitors, stimulatory compounds, or other factors may be added to the media at this time. For investigation of the impacts of specific compounds, each trigeminal ganglia is carefully bisected with a surgical scalpel. One half is incubated in the presence of the test compound while the other is incubated in the presence of control vehicle compound (paired ganglia explant test). The use of small molecule inhibitors specifically targeting cellular or viral factors has been useful in determining the role of these proteins in the transition from HSV-1 latency to lytic reactivation.

3. Place dish into a tissue culture incubator and incubate for the appropriate time parameter of the given experiment (1 hr to 48 hrs) at 37°C, 5% CO₂.

The act of explanting the trigeminal ganglion induces HSV-1 reactivation.

If compounds or test reagents are used during this incubation period, the media and compound should be replaced every 12–24 hrs (dependent upon the stability of the reagent).

4. At the appropriate time point, remove the trigeminal ganglion from the culture dish using curved-sharp tweezers.
5. Flash-freeze in liquid nitrogen or continue to Basic Protocol 4 or 5.

BASIC PROTOCOL 4

DETERMINING VIRAL TITER OF LATENTLY INFECTED TRIGEMINAL GANGLIA

In Basic Protocol 3 the experimental methodology of inducing reactivation through *ex vivo* explant of the latently infected trigeminal ganglion was described. One assay commonly utilized to assess the reactivation efficiency (stimulation or inhibition) is to determine the viral titer/yield 24–48 hrs post explant.

Quantitating the yield of viral progeny resulting from reactivation begins with homogenizing the tissue in culture media followed by brief sonication. Serial dilutions of these lysates are plated onto Vero cells and incubated to promote viral absorption and entry. A semi-solid matrix of agarose/DMEM is layered on top of the cells. After incubation, the resulting plaques are subsequently counterstained with neutral red such that living cells will uptake neutral red and thus appear red in color, while plaques (regions of dead cells) will appear clear (color of plastic dish). Plaques are counted and results are expressed as plaque forming units (PFU) per volume of lysate (typically expressed as PFU/mL) or PFU per ganglia.

Note: In addition to determining the viral yields in trigeminal ganglia, this protocol is also adaptable to other mouse tissues as well as tissue culture cells (see Support Protocol 1).

Materials—Vero cells (ATCC, Cat.# CCL-81)

PBS

0.25% Trypsin-EDTA

DMEM/5% FBS medium (see recipe)

12-well tissue culture dish(s)

HSV-1 infected mouse trigeminal ganglia (see Basic Protocols 2 and 3) or infected cell pellet (Support Protocol 1)

Glass dounce homogenizer (Wheaton, Cat #357844, 0.1 mL; note that this size will hold up to 1 mL)

DMEM/10% FBS medium (see recipe)

Sterile 1.5 mL tubes

Sonicator equipped with micro-tip probe

DMEM/1% FBS medium (see recipe)

42°C water bath

1% low melting agarose/DMEM/5% FBS medium (see recipe)

1% neutral red (see recipe)

Light box or inverted microscope

Plating Vero cells and homogenization of HSV-1 infected TGs—

1. Maintain a culture of Vero cells in DMEM/5% FBS. See **APPENDIX 4E** (Ammerman et al., 2008) for information regarding the growth and maintenance of Vero cell lines.
2. Plate sufficient numbers of 12-well plates containing Vero cells in order to complete duplicate wells for each titration.

On the day of the experiment, ensure 12-well dishes are ~100% confluent or 4×10^5 cells/well.

It is standard practice to make ~five 10-fold serial dilutions for each sample to be tittered.

Note: if you are titering infected tissue culture cells, proceed to step #6.

3. Transfer the trigeminal ganglion to a 0.1 mL glass dounce homogenizer with 500 μ l of pre-chilled DMEM/10% FBS medium (250 μ l for 0.5 ganglia in a paired ganglia explant experiment).

4. Disrupt ganglion in the dounce by grinding the ganglia against the sidewall using the glass pestle. Grind and twist the glass pestle until the ganglion is completely disrupted and the resulting solution is uniform. Transfer the solution to a sterile 1.5 mL tube and place on ice.
5. Repeat steps 3 – 4 until all ganglia have been dounced.

At this stage ganglia lysates can be flash-frozen in liquid nitrogen and stored at –80°C.
6. Using a sonicator equipped with a micro-tip probe, sonicate each sample for 10 to 15 sec (on ice) or until the tissue has been satisfactory disrupted.

Sonication conditions are dependent on the make and model of the sonicator. Optimal conditions for Branson Sonifer 450D are 15% amplitude, 1 cycle, 15 sec.

During sonication the probe may generate significant amount of heat. Sonication steps should be completed at 4°C with the 1.5 mL tube placed in an ice water bath.
7. Clarify the sonicated lysate by centrifuging for 5 min at 5000 rpm, 4°C. Transfer the supernatant to a sterile 1.5 mL tube.

If a relatively large pellet remains, optimize sonication conditions in step #6.

Dilution of ganglia lysate and infection of Vero cells—

8. Add 450 µl of pre-chilled DMEM/1% FBS to four 1.5 mL tubes. Prepare 10-fold serial dilutions by adding 50 µl of ganglia lysate to one of the 1.5 mL tubes containing 450 µl of DMEM/1% FBS and briefly vortex (10^{-1} dilution). Transfer 50 µl to a second tube containing 450 µl of DMEM/1% FBS and briefly vortex (10^{-2} dilution). Repeat dilution steps until 10^{-4} dilution is prepared.

Store diluted ganglia lysates on ice at all times.
9. Aspirate the media from the Vero cell cultures and pipette 0.2 mL of each dilution into duplicate wells. Transfer the 12-well dishes to the tissue culture incubator equipped with a rocker platform and allow the virus to absorb at 37°C for 2 hrs. The rocker should be set to slowly maintain a wet surface across the Vero cell monolayer.

Alternatively, if a rocker is not available during the viral adsorption stage, plates can be gently rocked every 20 min by hand to ensure equal distribution of the viral inoculum across the monolayer.

Agarose overlay—

10. During the absorption stage, prepare 1% low melting agarose/DMEM/5% FBS and allow to cool in a 42°C water bath (see recipe section).

11. Aspirate the inoculum from the Vero cells and gently overlay the monolayer with 1 mL of the prepared 42°C agarose/media. Maintain the plates at room temperature for 30 min or until the agarose has solidified.

Confirm that the agarose overlay medium is at the correct temperature. Temperatures above 42°C can disrupt absorbed virus and damage the cells. Conversely, if the agarose overlay is too cold, the agarose will solidify during the process of overlaying the culture. Avoid the addition of bubbles during the overlay process.

12. Once the agarose has solidified, gently add 0.5 mL of DMEM/5% FBS medium on top of the agarose.
13. Incubate the plates in a 37°C tissue culture incubator for 2 to 3 days.

Visualizing and counting of plaques—

14. Once plaques are visible, add 20 µl of a 1% neutral red stain solution to each well and carefully rock the plate to disperse the stain. Incubate the plates for an additional 4 to 6 hrs at 37°C.

Neutral red serves as a counter stain to discern individual plaques. Living cells will take up the neutral red, while plaques (regions of dead cells) will appear clear.

15. Remove the plates from incubator and carefully aspirate the top layer of medium without disturbing the agarose overlay.
16. Count the plaques using a light box or an inverted microscope. Only count wells in which plaques are easily discernable.

$$\text{pfu/mL} = (\text{Avg plaque \#})(\text{dilution})/\text{absorption volume in mL}$$

As an example, 34 and 40 plaques were counted from duplicate wells from a 10^{-3} dilution. Thus, the average number of plaques counted from a 10^{-3} dilution is 37, which can be represented as 3.7×10^4 pfu/0.2 mL. To express as pfu/mL, divide the titer by the absorption volume (0.2 mL) and the final titer is 1.85×10^5 pfu/mL.

BASIC PROTOCOL 5

IMMUNOHISTOCHEMICAL STAINING OF HSV-1 INFECTED TRIGEMINAL GANGLIA

Immunohistochemistry or immunofluorescence microscopy are powerful tools for the visualization of cellular and viral factors in the context of an infected tissue. The protocol below describes the immunohistochemical/immunofluorescent staining of mouse trigeminal ganglia for cellular and viral antigens. Experiments can probe for questions regarding the expression of viral proteins in the presence of small molecule inhibitors (see Basic Protocol 3) or the localization pattern of cellular and viral antigens during latency and reactivation (*ex vivo* explant).

Materials—Mouse trigeminal ganglia infected with HSV-1 (see BASIC PROTOCOLS 2 and 3)

Note that trigeminal ganglia can be removed and processed during stages of the initial lytic infection (1–14 days post infection) as well as during latency and reactivation stages.

4% paraformaldehyde in PBS (see recipe)

PBS

50%, 70%, and 95% ethanol in PBS (see recipe)

Metal slide-staining rack

Xylene

Hot plate

0.01M citric acid, pH 6.0, antigen unmasking solution (Vector laboratories, Cat.# H-3300)

Super-PAP pen

10% goat sera (Jackson Immuno Research, Cat.# 017–000–121)

Primary antibodies

Anti-Neurofilament N200 (Sigma-Aldrich, Cat.# N0142) -optional

Dark plastic box

0.02% Tween/PBS (see recipe)

Fluorescently conjugated secondary antibodies (Jackson Immuno Research)

DAPI Fluoromount-G (SouthernBiotech, Cat.# 0100–20)

Curved-sharp tweezers (Sigma-Aldrich, T4787, Style #7)

Glass coverslips (size: 30 × 24 mm, thickness: 0.13 to 0.17 mm)

Fixation of trigeminal ganglia—

1. Place trigeminal ganglia in 4% paraformaldehyde/PBS solution and incubate at 4°C for 4–12 hrs.
2. Aspirate the paraformaldehyde solution and replace it with 70% ethanol/PBS. Incubate at 4°C for ~12 hrs.

Note that the tissues can be maintained in this solution for up to 24 hrs prior to embedding.

3. Embed the tissue in paraffin and section the block with a microtome [see **UNIT 14D.4** (Venkatesh et al., 2013) and (Zeller, 2001) for basic principals of paraffin embedding and sectioning].

Ganglia are oriented in the paraffin during embedding to resemble an “open butterfly”. Sections of 4–8 μ m are cut onto Superfrost Plus microscope slides (Fisher Scientific, Cat.# 12–550–15).

Deparaffinization and hydration of sections—

4. At room temperature, place slides containing the sections in a staining rack filled with xylene for 3 minutes. Repeat 2 additional times with fresh xylene. Between each wash, drain the excess xylene from slides.
5. Repeat the washes using 95% ethanol (2×3 minutes each).
6. Incubate slides in 70% ethanol for 3 min.
7. Incubate slides in 50% ethanol for 3 min.
8. Remove slides from ethanol and place in PBS.

Store slides in PBS prior to antigen retrieval. From this point forward the sections are sensitive to drying. The drying of sections will cause tissue degradation and high background staining due to non-specific antibody binding. Process the slides as soon as feasible to prevent degradation of the tissue.

Antigen retrieval—

9. On a hot plate, heat 0.01 M citric acid, pH 6.0 to boil ($>96^{\circ}\text{C}$). Transfer the slides to the boiling citric acid solution and incubate for 10 min. Do not allow the temperature to fall below 96°C .

The method of antigen retrieval should be optimized for the utilized antibodies. Methods include citric acid method (described above) and proteolytic induced antigen retrieval. The citric acid antigen retrieval method has been effective for staining a wide range of cellular and viral antigens in the processed trigeminal ganglia.

10. Remove citric acid solution and slides from hot plate. Allow to slowly cool at room temperature until the solution is below 48°C .

Immunohistochemical staining—

11. Remove slides from citric acid solution and mark a water repellent circle around the tissue section using a Super PAP pen. This will maintain a boundary around the tissue and reduce the usage of primary and secondary antibodies.
12. Pre-block the section with 10% donkey serum in a hybridization chamber at room temperature for 1 hr.

A solution of 3% BSA can be used in substitution of 10% donkey serum depending on the level of background and the species in which the antibodies were generated.

Layering damp paper towels at the bottom of dark plastic box with a lid can be utilized as a hybridization chamber. Slides are placed on top of the damp paper towels to prevent the section from drying out.

13. Dilute the primary antibodies in 10% donkey serum.

The antibody dilution must be optimized for each antibody utilized. A solution of 3% BSA is sometimes used in place of the donkey serum when dictated by the specific antibody used.

The trigeminal ganglion is a network of neurons and support cells. It is recommended that sections be stained with a neuronal cell marker (Anti-neurofilament N200, Sigma-Aldrich, N0142) to differentiate neurons from support cells.

14. Carefully aspirate off the blocking solution and immediately pipette the diluted primary antibody onto the top of the section making sure to avoid bubbles. Incubate section in hybridization chamber at room temperature for ~2 hrs.

The optimal concentration of antibody and time of incubation must be empirically determined by the investigator.

15. Transfer slides to staining rack and place in a container filled with PBS. Dip the rack several times.
16. Transfer the rack with the slides to a new staining container filled with 0.02% Tween/PBS and incubate for 10 min.
17. Dip the staining rack several times in PBS.
18. Dilute fluorescently conjugated secondary antibodies in 10% donkey serum.

The antibody dilution must be optimized for each individual antibody utilized. As noted above, a solution of 3% BSA can be used in substitution of 10% donkey serum.

19. Remove the remaining PBS from the slides and carefully pipette diluted fluorescently conjugated secondary antibodies onto the top of the sections. Incubate the sections in dark hybridization box at room temperature for ~1 hr.

The optimal concentration of antibody and time of incubation must be empirically determined by the investigator.

Protect section from excessive exposure to light as this reduces the fluorescence signal during viewing of the section.

20. Remove the slides from the hybridization chamber and transfer them to the staining rack. Dip the rack several times in PBS.

21. Transfer the slides in the staining rack to a container of 0.02% Tween/PBS and incubate for 10 min.
22. Dip slides and staining rack several times in PBS.

Mounting of section—

23. Remove the remaining PBS from the slide and add 10–12 μ L of DAPI Fluoromount-G onto the top of the section.
24. Using tweezers, place one edge of the glass coverslip onto the slide and gently lay it over the section. Avoid making or trapping air bubbles.
25. Allow the slides to dry overnight in the dark at room temperature. For storage, place the slides at 4°C.

Slides are viewed using a fluorescence or confocal microscope with 63X to 100X oil immersion lenses [see UNIT 2C.1 (Smith, 2006) for basic principals of confocal microscopy and image acquisition].

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A** (Commonly Used Reagents); for suppliers, see **SUPPLIERS APPENDIX**.

Avertin, stock and working solution (20 mg/mL)—2,2,2-tribromoethanol (Avertin) (Sigma-Aldrich, Cat.# T-48402)

t-Amyl alcohol (Sigma-Aldrich, Cat.# A-1685)

PBS (without Ca⁺⁺ and Mg⁺⁺)

Sterile 0.2 μ M filter units

Stock solution: To prepare a stock solution of Avertin, add 1 mL of t-amyl alcohol for every 1 g of 2,2,2-tribromoethanol. Place on a rocker or shaker at room temperature until the Avertin is completely dissolved. Avertin is light sensitive and photo-oxidation products are toxic to the animals. Wrap the bottle with tinfoil or place in a dark bottle protected from light. The stock solution can be stored in aliquots at –20°C for up to 1 year. The Avertin will precipitate out of solution when thawing the stock aliquot and must be redissolved prior to dilution to the working solution. Important note: Use only freshly obtained stocks of tribromoethanol. The solid is unstable and will degrade to highly toxic products.

Working solution: To prepare working solution (20 mg/mL), dilute 0.5 mL of the Avertin stock solution dropwise in 39.5 mL of prewarmed sterile PBS (without Ca⁺⁺/Mg⁺⁺). Shake vigorously until completely dissolved. Wrap the tube of working solution with tinfoil to protect from light. Prepare the Avertin working solution just prior to beginning the infections. Discard any remaining unused working solution.

DMEM/10%, DMEM 5%, and DMEM 1% medium—Add the following to 500 mL of DMEM-high glucose (4.5 g/L)

6 mL of pen-strep (100X stock; 10,000 U/mL penicillin and 10,000 µg/mL streptomycin)

6 mL L-glutamine (100X stock; 200 mM)

50, 25, or 5 mL of heat-inactivated fetal bovine serum (FBS) for 10%, 5%, or 1%

Store up to 2 weeks at 4°C.

Low melting agarose 1% / DMEM / FBS 5%—Dissolve 1 g of low melting agarose (NuSieve GTG Agarose, Lonza, Cat.# 50080) in 50 mL of sterile PBS using a microwave oven. Place in a 42°C water bath and cool to ~50°C. Add 50 mL of DMEM/10% FBS and mix. Maintain the agarose/DMEM in the water bath to prevent premature solidification.

Neutral red, 1%—Dissolve 0.1 g of neutral red in <10 mL of PBS. Adjust volume to 10 mL with PBS. Store up to 6 months at room temperature.

Paraformaldehyde, 4%—Combine 10 mL of 16% (w/v) paraformaldehyde (Electron Microscopy Sciences, Cat.# 15710) with 30 mL PBS. Prepare fresh.

Tween-PBS, 0.02%—Combine 500 mL of PBS with 100 µl Tween-20. Prepare fresh.

COMMENTARY

Background Information—Primary infection with HSV-1 results in the productive replication of the virus at the initial site of infection (typically epithelial and fibroblast cells) that ultimately results in the production of progeny virus and the death of the infected cell (Roizman et al., 2007). Progeny virus may then infect local sensory neurons via dendrite projections at the site of infection. Within the neuronal nucleus, the viral genome enters the latency/quiescent state and may persist within the neuron for the life of the host.

This biphasic replication cycle, however, is characterized by cycles of reactivation from this latent state that can result in recurrent disease. Pathologies range from herpetic lesions to more severe cases of encephalitis and neurological issues attributed to congenital infections (Roizman et al., 2007; Whitley et al., 2007). Additionally, HSV-1 is the foremost cause of viral-mediated blindness in the developed world.

Nucleoside analogs such as acyclovir, which specifically target the viral DNA polymerase (UL30), are the predominant pharmaceuticals used to treat herpetic diseases. While effective, these drugs do not completely control subclinical viral shedding which is the major mode of transmission. Additionally, the clinical use of these compounds has resulted in the selection of resistant viral populations. Thus, understanding the molecular mechanisms involved in viral lytic and latency-reactivation cycles can perhaps reveal novel targets for the development of a new generation of antivirals.

During the lytic replication phase, there is a highly ordered cascade of viral immediate early, early, and late gene expression that leads to the maturation and synthesis of infectious viral progeny (Roizman et al., 2007). In contrast, latency is characterized by the lack of significant lytic viral gene expression and the predominant expression of the viral non-coding RNAs (LATs). However, mechanisms involved in the establishment, maintenance, and reactivation from latency remain unknown.

The mouse serves as an appropriate model of HSV-1 latency-reactivation to address these questions. This model involves corneal infection and the establishment of latency within the sensory neurons of the trigeminal ganglion. Stress-induced reactivation is simulated by isolation and explant of latently infected sensory ganglia that can be monitored by viral titrating (Basic Protocol 4), immunohistochemical or immunofluorescent staining (Basic Protocol 5), assessment of viral gene expression [see **UNIT 14E.5** (Turner et al., 2014)], or other molecular assays. Thus, the network of cellular and viral proteins regulating HSV-1 lytic infection, latency, and reactivation can be probed. The system is also amenable to the investigation of small molecule inhibitors or activators that target viral or cellular proteins and pathways to provide further mechanistic insights into the latency-reactivation cycle. Such studies have more recently highlighted the important role that modulation of the viral chromatin structure plays in the viral lytic and latency-reactivation cycles (Liang et al., 2013a; Liang et al., 2013b; Liang et al., 2009). These studies have also demonstrated the potential for the development of epigenetic pharmaceuticals as novel antivirals to control HSV disease.

Critical Parameters and Troubleshooting

Institutional approval of HSV-1 latency mouse model: One of the first critical parameters prior to establishment of the HSV-1 latency mouse model is the approval of animal protocols by the host research institute. It is important to carefully consider the parameters of the experiments, the value of the anticipated knowledge, and the appropriate ethical use of the animal population. Thus, a significant amount of time needs to be invested prior to the actual initiation of experiments.

The second key factor is the procurement of research funds and cage space within the animal research facility. The purchase of mice from distributors is a predictable cost. However, as infected animals are maintained for extended periods of time, there are also cage costs for maintenance of control and infected animals that require careful consideration.

Alternative HSV-1 latency models to consider: As for any animal model and experimental method, there are key features and alternative approaches to consider prior to the initiation of experiments. For the mouse model, the degree of HSV-1 spontaneous reactivation and shedding is low. In addition, the absence of corneal lesions makes this model less attractive for those specifically interested in ocular diseases. In contrast, the rabbit eye model exhibits a more robust level of spontaneous shedding, reactivation, and evidence of ocular disease (Webre et al., 2012).

Another important consideration is that the trigeminal ganglion is a tissue containing sensory neurons and a diverse population of support cells. Results must be interpreted in this

context. For some specific questions, an *in vitro* neuronal culture system with defined cell populations may be more appropriate (Baird et al., 2013; Bertke et al., 2011; Camarena et al., 2010; Hafezi et al., 2012; Markus et al., 2011; Wilcox and Johnson, 1987; Wilcox and Johnson, 1988).

Isolation of trigeminal ganglion: The isolation of the trigeminal ganglion from latently infected mice involves a degree of skill to remove the tissue, intact and undamaged, within a short period of time. Plans for appropriate training and practice should be made prior to the initiation of experiments.

Explant induced reactivation: One of the key components of inducing an efficient reactivation of HSV-1 is ensuring sufficient amount of virus has entered the eye during ocular infection. As described in Basic Protocol 2, careful scarification of the eye significantly enhances infection. However the efficiency and reproducibility of infection between animals is highly dependent on the level of scarification and the method applied by the particular investigator. Variables to consider are the number of scratches as well as the degree of pressure applied to the eye with the 30-gauge needle.

To simulate stress induce reactivation, ganglia are explanted into culture (see Basic Protocol 3) (Liang et al., 2013b; Liang et al., 2009). The investigator may optimize the duration of explant and the components of the explant media to optimize conditions for enhanced and synchronous reactivation. These parameters are important as a sufficient level of viral reactivation is required to quantitatively detect viral lytic mRNAs by quantitative real-time PCR [see **UNIT 14E.5** (Turner et al., 2014)] or the expression of viral proteins by immunohistochemical staining (see Basic Protocol 5). Several investigators have demonstrated enhanced reactivation when explant media is supplemented with anti-NGF antibody (Hill et al., 1997; Wilcox and Johnson, 1987; Wilcox and Johnson, 1988).

Anticipated Results

Course of ocular HSV-1 infection in mice: Ocular infection with herpes simplex virus will exhibit corneal lesions between 5 to 7 days with the peak of infection occurring between 7 and 14 days (Figure 5). During peak infection, corneal lesions may spread across the nose and facial area with mild to moderate fur loss. The animal may appear “scruffy,” however no significant change in weight should be observed (>20%). Approximately 10% of the infected animals may display symptoms of encephalitic infection (hunching, paralysis). Institutional guidelines and parameters of the investigator’s animal protocol will dictate intervention or euthanasia.

Between days 14 to 21, the mice will resolve the infection. Mice may exhibit minor fur loss across the nose and facial area, while lesions will scab and clear. The animals should remain active. To ensure establishment of latent HSV-1 infection, mice are caged for 4–8 weeks post resolution of infection prior to isolation of trigeminal ganglia (see Basic Protocol 2).

Ex vivo explant reactivation and use of small molecule inhibitors: To stimulate viral reactivation, trigeminal ganglia are explanted and cultured. Explant of the trigeminal ganglion induces various stress pathways within the cell that ultimately leads to initiation of

the lytic replication cycle and the production of infectious progeny virus. Typical viral titers isolated under the current conditions detailed in this chapter range from 10^3 to 10^6 PFU per ganglia.

The *ex vivo* explant model has been utilized for studies pertaining to factors essential for lytic replication and reactivation from latency (Basic Protocol 3). The use of small molecule inhibitors and activators that target cellular pathways including chromatin modulation machinery that impact the state of the virus have been successfully utilized (Liang et al., 2013a; Liang et al., 2013b; Liang et al., 2009).

Immunohistochemical staining of latently infected TGs: The immunohistochemical (IHC) staining of trigeminal ganglia from HSV-1 latently infected mice is a powerful tool for the visualization of cellular and viral factors in the context of an infected tissue. The methods described in Basic Protocol 5 have been successfully utilized for localization studies of cellular and viral proteins essential for reactivation. As shown in Figure 6a, the cellular protein Host Cell Factor-1 (HCF-1), an essential component of HSV-1 coactivator complex for lytic gene expression, predominantly localizes to the cytoplasm of unstimulated neurons. However during reactivation (*ex vivo* explant), HCF-1 is rapidly transported to the nucleus. This shift in the localization promotes assembly of the viral coactivator complex, which epigenetically modulates the chromatin status of the viral genome to stimulate the expression of lytic viral genes (Knipe and Cliffe, 2008; Kristie et al., 2010).

Additionally, the status of viral reactivation can be monitored by IHC/IF visualization of primary neurons and support cells undergoing lytic viral reactivation. This represents a quantitative method for assessing the individual neurons expressing lytic antigens which can be used to determine the impacts of perturbations of the reactivation process using small molecule inhibitors. For example, inhibition of chromatin modulation components linked to the HCF-1 coactivator complex, such as the histone demethylases LSD1 or the family of JMJD2s blocks reactivation from latency as evident by the reduced number of primary neurons staining for the viral lytic replication protein ICP8 (U_L29) (Figure 6b) (Liang et al., 2013b; Liang et al., 2009).

Time Considerations—See TABLE 1 for time consideration for all protocols discussed.

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Figure 1. One-handed method of restraining mice

With the opposite hand, gently but firmly grab the skin at the back of the neck with thumb and forefinger. Pull gently on the tail to stretch the animal and secure the tail with pinkie finger. At this stage the mouse should be comfortably but firmly restrained with one hand.

Figure 2. Method of ocular scarification

(A) With your thumb and index finger, gently press down on the skin surrounding the eye until the eye protrudes from the socket (B). (C) To scarify the eye, gently stroke 4 – 5 times across the eye making sure that the bevel of the 30-gauge needle is facing up.

Figure 3. Preparation and dissection of mouse cranium

(A) Instruments utilized in dissection (left to right): forceps, iris/curved scissors, surgical pins, half curved eye dressing forceps, curved-sharp tweezers, and micro-dissecting spring scissors. (B) Insertion of surgical pins into the nasal cavity and hindquarters of a euthanized mouse. (C) Removal of skin tissue with iris/curved scissors to expose the skull. (D) Holding the posterior of the skull with half curved eye dressing forceps and cutting the top circumference of the skull.

Figure 4. Isolation of trigeminal ganglia

(A) The brain (arrow) is exposed following removal of the skull. (B) Once the brain is removed, detach the bridge of the eye socket to display the anterior portion of the trigeminal ganglion. (C) Detailed comparison of trigeminal ganglia with the eye socket bridge removed (bottom) and intact (top, arrow). (D) Paired trigeminal ganglia (arrows) exposed. (E) Gently lift the trigeminal ganglion with curved-sharp tweezers and cut the neuronal projects that are emanating from the base of the skull. (F) An isolated trigeminal ganglion.

A **B** **C** **D**

Figure 5. Course of ocular HSV-1 infection in mice

Ocular infection with HSV-1 will exhibit (A) corneal lesions between 5 to 7 days. During peak times of infection (7 to 14 days), (B) corneal lesions may spread across the nose and face. Between days 14 to 21, the mice should resolve the infection. The mice may exhibit (C) minor fur loss across the nose and face, while (D) lesions will scab and clear.

Figure 6. Immunohistochemical staining of mouse trigeminal ganglia

(A) Trigeminal ganglia were either immediately fixed with paraformaldehyde or induced (see Basic Protocol 3) by explanting prior to fixation. At time 0, HCF-1 (green) predominantly localizes to the cytoplasm of neurons (red). However during explant, HCF-1 is localized to the nucleus (blue, DAPI). (B) Latently infected trigeminal ganglia were explanted in the presence of a JMJD2 inhibitor (ML324) or with DMSO (vehicle) (Liang et al., 2013b). Inhibition of the activity of the JMJD2 family reduces the number of neurons

undergoing HSV-1 reactivation from latency [ICP8 (red)]. Neurofilament (green), nucleus (blue, DAPI).

Table 1

Time considerations for HSV protocols

Protocol	Operation	Time required
Basic Protocol 1	Acclimation of mice to environment	1 week
Basic Protocol 1	Ocular infection of mice	3 hrs
Basic Protocol 1	Establishment of HSV-1 latency in mice	8 weeks
Basic Protocol 2	Isolation of trigeminal ganglion	2 hrs
Support Protocol 1	Preparing virus stocks	5 – 8 days
Basic Protocol 3	Induced HSV-1 reactivation	1 – 48 hrs
Basic Protocol 3	Incubation of small molecule inhibitors, antibodies, inhibitory or stimulatory compounds during HSV-1 reactivation	1 – 48 hrs
Basic Protocol 4	Determining trigeminal ganglion viral titer	6 days
Basic Protocol 5	Immunohistochemical staining	2 days