

**DEVELOPMENT OF PRIMARY NEURONAL CULTURE OF EMBRYONIC RABBIT DORSAL ROOT
GANGLIA FOR MICROFLUIDIC CHAMBER ANALYSIS OF AXON MEDIATED NEURONAL SPREAD
OF BOVINE HERPESVIRUS TYPE 1.**

by

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B.A., University of Kansas, 2005

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2010

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Abstract

Bovine herpesvirus type 1 (BHV-1) is an important pathogen of cattle that can cause severe respiratory tract infection known as infectious bovine rhinotracheitis (IBR), abortion in pregnant cows, and is an important component of the Bovine Respiratory Disease Complex (BRDC, "Shipping fever"). The ability of BHV-1 to transport anterogradely from neuron cell bodies in trigeminal ganglia to axon termini in the nasal and ocular epithelia of infected cattle complicates the control of the disease in both vaccinated and infected cattle populations. In calves and rabbits, Us9 deleted viruses have defective anterograde neuronal spread from cell bodies in the trigeminal ganglia to nerve termini in the nose and eye but retrograde spread remains unaffected. To characterize the neuronal spread of BHV-1, we developed primary neuronal cultures using the dorsal root ganglia (DRG) of rabbit embryos. We successfully used microfluidic chamber devices to isolate DRG in the somal compartment and allowed for efficient growth of axons into the axonal compartment. This enabled us to study axon mediated neuronal spread of infection as well as viral transport in axons. Thus, rabbit DRG neuronal culture was susceptible to BHV-1 mutant and wild-type infection, and the method allowed visualization of viral spread in chamber cultures using live cell imaging and fluorescent microscopy. Lastly, using the microfluidic chamber compartmentalized neuron culture system we showed that Us9 acidic domain-deleted and Us9 null mutant BHV-1 viruses had defective anterograde neuronal transport relative to BHV-1 wild type and/or Us9 rescued viruses.

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Acknowledgements

I would like to thank my major professor and advisor Dr. Shafiqul I Chowdhury for all of his guidance and support throughout my research. I would also like to thank Sara Navarro (technician) and Becky Neis (summer research student) for their contributions in establishing the primary neuronal culture system. My research was funded by Dr. Chowdhury's USDA grant # 35204-05200.

CHAPTER 1 – Introduction

Bovine herpesvirus type 1 (BHV-1) is an important pathogen of cattle that can cause severe respiratory tract infection known as infectious bovine rhinotracheitis (IBR), abortion in pregnant cows, infectious postular balanoposthitis, infectious pustular vulvovaginitis and is an important component of the Bovine Respiratory Disease Complex (BRDC, “Shipping fever”)(Wyller 1989). BRDC costs the U.S. cattle industry significant economic losses and is considered the primary health concern of the feedlot industry.

During primary infection, BHV-1 replicates in the nasal epithelium causing rhinotracheitis and enters the sensory nerve endings (axon termini) of the trigeminal nerve in the nasopharynx (Jones 2007). Viral transport from epithelium to neuronal cells involves cell-to-cell spread from the epithelial cells to the axon termini (Wang 2005). Nucleocapsids in conjunction with tegument structures are then transported retrogradely (up the axon, towards the cell body) to the trigeminal ganglion (TG) where they establish life-long latency (Rock 1986, Jones 2003). Throughout latent infection in the sensory ganglia, latency associated transcripts (LAT) and their corresponding proteins have been detected in the sensory ganglia, although viral replication cannot be detected nor can virus be isolated (Jiang 1998). During stages of stress or corticosteroid administration the latent virus reactivates in the cell bodies of the trigeminal ganglia and commonly transports anterogradely down the axon towards the peripheral nerve endings in the nasopharynx (maxillary branch of the trigeminal nerve) and cornea (ophthalmic branch of the trigeminal nerve) and subsequently virus is shed (Chowdhury 2000, Butchi 2007, Enquist 1999, Smith 2002, Jones 2007).

Herpesviruses contain four major structural components; the envelope, capsid, tegument, and genome. The envelope is the outermost portion of the virus which contains various host lipid components comprised of the cytoplasmic membrane portions as well as the nuclear membrane. The envelope surrounds the viral capsid which is an icosahedral shell of 150 hexons and 12 pentons (Roizman 2001). The envelope is coupled to the capsid by way of the tegument. The genome is the inner most structural component which consists of a linear molecular of double stranded DNA. The bovine herpesvirus type 1 genome consists of about 140kb and is comprised of two unique regions; the short unique region (US) and the long unique region (UL) (Chowdhury 2000). The BHV-1 genome is very large and encodes around 70 proteins. Herpesviruses encode a complete replication system which includes a viral DNA dependent DNA polymerase and enzymes that synthesize DNA precursors. However, herpesviruses must initially use the cellular enzyme DNA-dependent RNA polymerase to transcribe DNA into RNA (Roizman 2001).

Alphaherpesviruses, encode at least eleven envelope glycoproteins (Enquist 1999, Mettenleiter 1994). Five glycoproteins; gB, gD, gH, gL, and gK are essential because they are required for replication in tissue culture (Van Drunen Littel-van den Hurk 1986). Six glycoproteins; gC, gE, gG, gI, gM, gN and one non glycosylated protein (Us9) are non-essential because they are not required for growth in tissue culture (Van Drunen Littel-van den Hurk 1986, Mettenleiter 1991). Glycoprotein C homologs in alphaherpesviruses facilitate initial attachment of virus to the cell surface by binding to heparan sulfate proteoglycan (Enquist 1999, Mettenleiter 2002). Glycoproteins gB, gC, gD and gH/gL of BHV-1 and their homologs in

other alphaherpesviruses are important for virus entry into cells and glycoprotein gK is important for viral release in cell culture (Koshizuka 2005, Foster 1999, Mettenleiter 1994).

Herpesvirus infection of the neuron in the trigeminal ganglia begins with viral particles entering sensory nerve endings following infection of respiratory and ocular epithelial cells. Like in non neuronal cells, the entry process in neurons is mediated by glycoproteins gB, gC, gD and gH/gL (Curanovic 2009). Upon encountering an axon terminal, herpesvirus envelope fuses with the cell membrane and the capsid along with tegument proteins are introduced into the cytosol (Mettenleiter 1994, Brideau 2000). Once in the cytosol, wild-type alpha herpesvirus capsids along with tegument proteins are transported retrogradely to the neuronal cell bodies in the trigeminal ganglia (TG) where they establish latency (Chowdhury 2000, 2006). The retrograde transport involves dynein motor-mediated, fast axonal transport of capsids and tegument from plus to minus ends of the microtubules, toward neuronal cell bodies and nuclei in the ganglia where latency is established (Antinone 2006, Smith 2002).

Upon reactivation, viral DNA replicates and produces virion components in the nerve cell body which localize to the axon and then progress anterogradely towards axon termini (Lyman 2007, Wang 2005, Brideau 2000). The anterograde transport involves, kinesin family motor-mediated fast axonal transport of vesicles containing partially or fully enveloped viruses from minus to plus ends of the microtubules toward the nasal and corneal epithelium (Smith 2002). The location of virus assembly within the neuron is controversial and currently two models exist that describe axonal transport (Antinone 2006). The separate model suggests that viral glycoproteins, tegument proteins and nucleocapsids are transported separately from the cell body to the axon termini where they assemble prior to virus spread to the post synaptic

neurons. The other model holds that viruses transported anterogradely along the axon are fully enveloped (Antinone 2006). Despite these controversies, it is well accepted that Us9 and gE/gI play crucial roles in axonal transport of viruses, from the cell body to the axon. This leads to nasal and ocular virus shedding and subsequent virus transmission to susceptible animals (Jones 2003). Therefore, retrograde and anterograde transport mechanisms are crucial for the maintenance and transmission of BHV-1 in the susceptible animal population.

BHV-1 envelope proteins; glycoprotein gE and unglycosylated glycoprotein Us9 are involved in anterograde neuronal transport (Butchi 2007, Chowdhury 2002, 2006, Tomishima 2001). In alphaherpesviruses including BHV-1, BHV-5 and PRV envelope glycoproteins gE, gI and Us9 are required for anterograde axonal transport and trans-synaptic spread from primary neurons to secondary post synaptic neurons but are not required for retrograde transport from postsynaptic to presynaptic neurons (Brideau 2000, Chowdhury 2002, Butchi 2007). Contrary to BHV-1 and PRV requirement of Us9, gE, and gI for anterograde transport, recent reports in herpes simplex virus type 1 show that anterograde transport of virus requires gE and gI but not Us9 (McGraw 2009).

The proteins associated with retrograde transport are tegument proteins essential to the viral life cycle; if these proteins are manipulated, the virus is no longer viable (Smith 2002). However, both glycoprotein E (gE) and Us9, may be deleted without affecting retrograde transport of the virus (Enquist 1999; Mettenleiter 1994, Osterrieder 1999). In calves and rabbits, both BHV-1 gE and Us9 deleted viruses have defective anterograde neuronal transport from cell bodies in the trigeminal ganglia to axon termini in the nose and eye (Chowdhury 2002, 2006, Butch 2007, Liu 2008). Pseudorabies virus (PRV) Us9 null mutants also exhibit defective

anterograde neuronal spread (Brideau 2000, Tomishima 2001, Lyman 2007). Our lab has also found that BHV-1 Us9 83-90 acidic domain-deleted mutant virus had defective anterograde neuronal transport in vivo (Chowdhury manuscript in progress).

Alpha-herpesvirus Us9 homologs including BHV-1 Us9 are non glycosylated type II membrane proteins with a long cytoplasmic domain and a short ecto-domain (Chowdhury 2006, Lyman 2009). BHV-1 Us9 open reading frame is predicted to contain 144 amino acid residues with 111 amino acid long N-terminal cytoplasmic domain followed by a 29 amino acids spanning the lipid bilayer membrane and a 4 amino acid long carboxy terminal extracellular/ecto domain (Chowdhury 2006, Butchi 2007). The Us9 cytoplasmic domain contains an acidic domain (amino acids 79-98) and a stretch of positively charged arginine residues (amino acids 104-111) immediately upstream of the transmembrane domain (Chowdhury 2006). Within the acidic domain, there is a predicted tyrosine phosphorylation motif and several potential casein kinase II phosphorylation sites (Chowdhury 2002, 2006). In addition, there are several serine and threonine residues that are potential phosphorylation sites. BHV-1 Us9 is heavily phosphorylated, both in infected cells and virions and has an apparent molecular mass of 30-32 kD (Chowdhury 2006).

Viral assembly in epithelial and neuronal cells is known to occur in the trans-golgi network (Lyman 2009). Us9 protein exhibits steady-state localization in or near the *trans*-Golgi network (Brideau 2000). Neurons are highly polarized cells and like in polarized epithelial cells biosynthetic post-Golgi trafficking is directed either to dendrites (similar to basolateral surfaces) and/or to axons (similar to apical surface) (Dotti 1990). Sorting mechanisms in the neurons however are not well characterized. In neurons, the axonal targeting machinery (equivalent to

apical surface) can utilize sorting signals within the cytoplasmic tail instead of apical motifs located within the transmembrane and ectodomain (West 1997). Currently, the mechanism of BHV-1 viral anterograde transport specifically post-Golgi glycoprotein transport is not well understood.

Us9 acidic domain motifs are highly conserved among the alpha herpesvirus Us9 protein homologs including herpes simplex virus, varicella zoster virus, pseudorabies virus, bovine herpesvirus and equine herpesvirus (Chowdhury 2006, Lyman 2009). Accumulating evidence suggests that Us9 acidic domains have an important function in the anterograde spread of herpesviruses (Brideau 2000, Favoreel 2006, Lyman 2008). Brideau et al reported that mutating tyrosine kinase phosphorylation and casein kinase phosphorylation sites within the Us9 acidic domain region dramatically reduced anterograde spread of infection in the rat brain model (2000). Lyman et al reported that primary amino acid sequences and conserved domains of PRV and BHV-1 Us9 proteins are highly conserved (2009). Amongst the veterinary homologs; PRV-1, EHV-1, and BHV-1 serine and tyrosine residues are highly conserved (Lyman 2009). Mutation of two tyrosines and two serines within the acidic domain of PRV Us9 hindered axonal sorting in dissociated primary neuronal cultures similar to previous results in the rat brain (Brideau 2000, Lyman 2009). Changing the two conserved tyrosines to alanines or mutation of two serine residues terminated the ability of axon mediated anterograde spread in PRV (Lyman 2009). Lyman et al also showed that BHV-1 Us9 homolog's functionally compensated for the loss of PRV Us9 in transneuronal spread (Lyman 2009). The BHV-1 Us9 83-90 acidic domain region contains key serine and tyrosine residues analogous to PRV. Preliminary results in our lab showed that BHV-1 Us9 83-90 acidic domain-deleted virus had defective anterograde transport

in calves and rabbits because no subsequent virus shedding was documented following reactivation from latency (Chowdhury manuscript in progress). To obtain direct evidence of defective anterograde spread, the microfluidic chamber system will demonstrate the importance of these acidic domain regions of BHV-1.

It is well accepted that pseudorabies virus Us9 protein targets viral capsids and glycoproteins to axons of dissociated sympathetic neurons (Lyman 2007). Neuroscience research has shown that neurons sort a subset of axolemmal proteins by a mechanism that requires the formation of protein-lipid rafts (Ledesma 1998). Lipid rafts are specific microdomains of apical membranes of epithelial cells or the similar apical membrane of neurons, which are the axon termini, and contain sphingolipids and cholesterol (Lyman 2008). Recent studies have suggested that Us9 protein mediated sorting and targeting of viral proteins into the axon during anterograde spread is due to the association of Us9 with lipid rafts (Lyman 2008). Favoreel et al also reported that at least four major PRV glycoproteins; gB, gC, gD, and gE are associated with lipid rafts due to their coaggregation together with monospecific antibodies on the plasma membranes of infected cells of swine epithelial cells (2004). Phosphorylation of Us9 is not required for lipid raft association but likely occurs after entering the raft complex and is vital for axonal sorting (Lyman 2008). Us9 protein lipid raft association has yet to be elucidated in BHV-1.

The advent of microfluidic devices has created various methods to study complex biological systems for analysis of cellular microenvironments (El-Ali 2006, Breslauer 2006). Microfluidics is a multidisciplinary field of study utilizing devices with small fluid channels that have precise control and the ability to manipulate small amounts of fluid (Park 2008). Initially

microfluidic devices were very difficult for bench top scientists to use because they were fabricated from etching on glass and in silicon (El-Ali 2006). Recently, fabrication of microfluidic devices in poly(dimethylsiloxane) (PDMS) by soft lithography have allowed for greater use in biomedical research (McDonald 2000). Microfluidic devices have been further specified for use in neuroscience research of stem cells, cell migration, and neurons (Liu 2008).

In these culture devices, neuronal cell bodies are located in the somal compartment and their axons project through microcapillary grooves to the opposing axonal compartment. The system uses a fabricated PDMS (polydimethylsiloxane) polymer positioned on a glass coverslip. The microgrooves provide a barrier that allows fluidic isolation of neuronal cell bodies but allows neurite outgrowth from the somal to the axonal compartment, where detector cells can be grown (Liu 2008). Fluidic isolation also creates a hydrostatic pressure differences between each respective compartment that allows for isolation of virus in either the somal or axonal compartment (Park 2006).

To show the role and understand the mechanism of BHV-1 Us9 protein in anterograde neuronal transport, tracing of virus and viral particles within intact neurons is necessary. However, it is difficult to trace a virus *in vivo* within a neuron. Therefore, the characterization of neuronal transport of BHV-1 mutants requires an *in vitro* neuronal culture system. Protocols for dissecting and culturing primary neurons from rats and mice are well established in various animal models (Ch'ng 2006, Park 2006). Interestingly, a method of establishing primary neuronal culture in fetal rabbits has not yet been published. Goldenberg et al. first described methods for culturing primary rabbit dorsal root ganglia for use in neuroscience research (1982). Among common laboratory animals, the rabbit is the only species susceptible to BHV-1,

so we sought to develop our cultures from neurons of the dorsal root ganglia (DRG) of rabbit embryos (Abril 2004, Chowdhury 2007, Rock 1982). We hypothesized that the rabbit dorsal root ganglia sensory neuronal culture will be susceptible to BHV-1 mutant and wild-type infection based on previous in vivo experiments with calves and rabbits.

The objectives of this project were to establish microfluidic chamber cultures using primary rabbit dorsal root ganglia. Once we established the culture system we used the platform to study the neuronal transport and importance of BHV-1 Us9 deleted, BHV-1 Us9 83-90 acidic domain deleted, and BHV-1 Us9 83-90 rescued virus in the microfluidic chamber culture system.

CHAPTER 2 – Materials and Methods

Virus Strains and Cell Lines

BHV-1 Cooper (Colorado-1) strain, acquired through American Type Culture Collection, was used in all experimental studies. BHV-1 strains included previously constructed viruses; BHV-1 Us9 83-90 BAC rescued, BHV-1 Us9 83-90 BAC deleted, BHV-1 Us9 deleted harboring GFP (Chowdhury 2006, Chowdhury manuscript in progress). All virus strains were cultured in Madin-Darby bovine kidney cells (MDBK) supplemented with 2% inactivated fetal bovine serum (Hyclone, SH30070.03) and 1% penicillin and streptomycin (P/S) (Lonza 17-603E) antibiotics.

All viruses were propagated and titrated in Madin-Darby bovine kidney (MDBK) cells as previously described (Chowdhury 1996). The MDBK cells were maintained in the laboratory with medium containing 5% inactivated fetal bovine serum and 1% P/S antibiotics. Cell culture flasks were used to passage the cells every three-four days with a trypsin washing and subsequent trypsin inactivation for five minutes at 37°C. Cells rounded up and detached after trypsin incubation and resuspended in fresh medium. All cells were maintained in a humidified 5% CO₂ air atmosphere incubator at 37°C.

Virus Stock

Viruses were propagated in Falcon T-150 cell culture flasks with a multiplicity of infection (MOI) of 0.01 (one infectious particle for every 100 MDBK cells). Confluent T-150 cell culture flasks contain approximately 2×10^7 cells. Infection was initiated when the flasks contained a confluent monolayer of MDBK cells. The viruses were serially diluted as need be in

2% IFBS and 1% P/S antibiotics DMEM media. The flasks were gently rocked every 15 minutes for a total of one hour to encourage virus attachment and absorption throughout the entire flask. Following absorption the infected MDBK cells were incubated at 37°C in a 5% CO₂ incubator. When cytopathic effect (CPE) reached 90% or greater the cells were placed in the -80°C freezer. Cells were briefly thawed and aliquoted into sterile vials stored at -80°C.

Preparation and Assembly of Microfluidic Polydimethylsiloxane (PDMS) Chambers

Protocols and fabrication of the microfluidic chamber system have been previously described but modified for our purposes (Park 2006, Liu 2008). Microfluidic chambers with microgroove length of 450 µm and a width of 10 µm were used (Xona Microfluidics SND450). In preparation glass coverslips were coated with 500 µg/mL poly-dl-ornithine (Sigma P8638) overnight in a humidified 5% CO₂ incubator at 37°C. The following day the poly-dl-ornithine was aspirated from the dishes and coverslips were washed five times with tissue culture grade water. The glass coverslips were dried in the tissue culture hood for no less than 30 minutes while the microfluidic chambers were sterilized in 95% ethanol and allowed to dry. The chamber was then placed onto the glass coverslip until it properly sealed. 10µg/mL of natural mouse laminin (Invitrogen 23017-015) was added to the microfluidic chambers and repeated pipetting of laminin was performed in order to encourage the fluid to cross the microgrooves of the chambers. All of the compartments were filled completely with laminin and the chambers were incubated overnight as described previously. The following morning laminin was aspirated from the dishes/chambers and washed twice with HBSS CMF. Neurobasal media (Gibco 21103) supplemented with 100ng/ml of nerve growth factor 2.5 S (Invitrogen 13257-019), B27 (Gibco

17504), 1% of penicillin and streptomycin was added immediately to the microfluidic chambers and placed back in the incubator. All of the chambers remained in the incubator until the neurons were added.

Animal Surgery

New Zealand myrtle white rabbits received on the 26th day of gestation were used for dissection the following day (27th day of gestation) from (Charles River Laboratories). The pregnant rabbit was anesthetized by isoflurane mask induction and subsequently euthanized with intracardial Phenobarbital injection. All protocols have been approved by The Institutional Animal Care and Use Committee of Louisiana State University protocol # 08-075. In preparation for hysterotomy, the female rabbit was placed in dorsal recumbency, the ventral abdomen was clipped and cleaned with alcohol and a ventral midline incision was made along the linea alba through the skin, tunica muscularis and subsequent abdominal muscles to expose the abdominal contents. The uterine horns were exteriorized and each horn was subsequently incised. Each embryonic rabbit kit was removed and placed in Hanks buffer containing calcium and magnesium (HBSS) (Sigma H6648).

The kits were placed into a second wash of HBSS and placed into the culture hood for dissection. The kits were dissected and the ventral portion of the spinal column removed to reveal the spinal cord and the dorsal root ganglia. We concentrated on removing ganglia from the cervical region of the spinal cord but occasionally removed thoracic ganglia. Approximately 80 cervical dorsal root ganglia were harvested from 8-12 fetuses. The ganglia were placed into one well of a six well tissue culture dish containing HBSS on ice.

Dissection and Dissociation of Rabbit Dorsal Root Ganglia

The ganglia were examined under dissecting microscopes to remove nerve rootlets and iris scissors were used for further dissociation. After careful removal, the ganglia were placed into a 15mL conical centrifuge tube and allowed to settle down, and then the supernatant was removed. The remaining ganglia were washed with 2mL Hanks calcium magnesium free medium (HBSS CMF) (Sigma H6648) and centrifuged in a Beckman-Coulter table top centrifuge at 4°C for 2 minutes at 2000rpm (931xg). Use of calcium and magnesium free Hanks buffer is imperative for best trypsinization conditions. The centrifugation was repeated once more and the ganglia were removed from the 15mL tube and placed into a 2mL Eppendorf tube.

The first step of dissociation was carried out by adding 1ml of 5000U/ml pre-warmed collagenase solution (Worthington 4214) to the pelleted ganglia and placed at 37°C for 20 minutes. The pellet was triturated 3-5 times with a 1mL Eppendorf cut pipette tip coated with the 2% sterile BSA. The ganglia were centrifuged and the supernatant removed as described previously. Careful removal of the supernatant is imperative in order to remove all residual collagenase, since the collagenase was not inactivated. The pellet was washed once with HBSS CMF and centrifuged as described previously. Next the ganglia were incubated for 15 minutes at 37°C in 250 µg/mL trypsin (Worthington LS003736). 100µL of inactivated fetal bovine serum was added to the 1mL pellet to make a final concentration of 10% and incubated for 5 minutes at 37°C in order to inactivate the trypsin. The ganglia were centrifuged as described previously and suspended in 500µL-1000µL of Neurobasal Media (Gibco 21103) containing 2 mM of L-glutamine, B27, 1% streptomycin/penicillin and 100ng/mL of nerve growth factor 2.5S (Invitrogen 13257-019). Flame smoothed Pasteur pipette tips coated with a sterile 2% BSA in

PBS solution were used to triturate the ganglia before plating. Coating prevents ganglia from attaching to the inner surface of the pipette and complete trituration removes clumps from the solution.

Plating Dissociated Neurons

The dissociated ganglia were suspended accordingly in supplemented neurobasal media. Media was removed from each microfluidic chambers somal compartments with around 100 μ L remaining in each of the axonal reservoirs. 20 μ L of neuronal ganglia suspension was loaded into the somal compartment on each side and placed into the incubator for 45-60 minutes. The neurons attached to the laminin substrate during this time and then an additional 100 μ l and 50 μ l of neuron culture media was added into each well of the somal and neurite reservoirs of the microfluidic chambers.

Maintenance of Neurons

48 hours after plating the neurons 1 μ M of cytosine β -d-arabinofuranoside (Ara-C) (Sigma C6645) was used to inhibit and eliminate growth of non neuronal cells. Twenty four hours post Ara-C treatment, the media in the somal chamber was replaced with 150 μ l of neuron culture media and the media in the neurite chamber was replaced with 150 μ l neurite promoting media (neuron culture plus neurite media) which contained additional neurite growth promoting factors: BDNF 10 ng/ml (Invitrogen 10908-010), CNTF 10ng/ml (Invitrogen PRC7015) and 10ng/ml GDNF (Invitrogen PHC7045). Cultures were maintained in the humidified 37°C and 5% CO₂ incubator until subsequent media changes were needed. It is best

to change media every two-three days to maintain physiological pH of the media. The media was changed every day if needed when the color of the phenol red pH indicator was too acidic.

Virus Infection

After 12-15 days the axonal compartment had dense neurite outgrowth in the microfluidic chambers. The chambers are then infected with virus to detect viral spread in the anterograde or retrograde direction. A variety of BHV-1 Us9 mutants and wild type rescued viruses were used for infection. Before viral infection Madin-Darby bovine kidney cells (MDBK) were plated into the axonal compartments of the respective chambers. The area of a microfluidic chamber axonal reservoir compartment was calculated and compared to the area and cell density of a T-25 flask. MDBK cells were added to the axonal compartment and formed a confluent single monolayer.

Anterograde infection of nerve cell bodies in somal compartment was performed 24 hours after addition of MDBK cells. The amount of viral inoculums added was estimated to be 1.5×10^7 plaque forming units (PFU) per chamber. 150 μ L of virus inoculum was added to each respective somal compartment of the microfluidic chambers. Neurobasal media with growth factors was replaced in the axonal compartment with 200 μ L of media per reservoir. The gradient created hydrostatic pressure that prevented diffusion of viral particles from the somal compartment to the axonal compartment. The chambers were placed into the humidified incubator at 37°C for at least 24 hours or until sufficient CPE had taken place.

For retrograde infection of axon termini in the neurite compartment the same procedure in reverse order was completed as above. First 200 μ L of neuron culture media was

added to the each somal compartment and 150 μ L of virus inoculum was added to each respective axonal compartment of the microfluidic chambers. The chambers were then incubated and processed as above.

Media was removed from all microfluidic chambers and stored at -80°C for later titration as described previously. Microfluidic chambers were washed once with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 8 minutes, and fixative was removed with two subsequent washes with PBS. The fixative was both added directly to the microfluidic chamber reservoirs or the chamber was removed and added directly to the cells. Cells were then incubated with permeabilization solution containing 3% BSA for 10 minutes. After permeabilization the three standard washes with PBS were performed.

Indirect Immunofluorescence Assays and Microscopy

Before incubation with primary antibody, cells were incubated with PBS containing 3% bovine serum albumin for 30mins to reduce background. The primary antibody, Rabbit α Neurofilament 200 (N 4112 Sigma), was diluted 1:1000 in 1x Tris buffered saline (TBS) and incubated for one hour at room temperature. After the one hour incubation the primary antibody was removed and cells washed three times with TBS. Next, secondary antibody, Alexa fluor 594 Donkey anti Rabbit (A-21207 Invitrogen) diluted 1:500 in 1x TBS, was added to each chamber for 20 minutes and incubated in the dark. After incubation the antibody was removed and chambers subsequently washed three times with TBS. The samples were either stored at 4°C or washed once with water and visualized. Microscopy was performed using an Olympus inverted microscope equipped with a camera. Live cell imaging with an Olympus IX71 inverted

microscope equipped with fluorescence illumination was also used to detect green fluorescent protein emission from the mutant viruses used in the microfluidic system.

CHAPTER 3 – Results

Rabbit Dorsal Root Ganglia Provide Viable Neurons For in Vitro Use

Embryonic rabbit dorsal root ganglia provided viable neuronal cell bodies for in vitro growth in a microfluidic chamber culture system. Approximately 80 cervical dorsal root ganglia were harvested from 8-12 embryonic rabbits on the 27th day of gestation. Ganglia were subsequently incubated with a collagenase solution and also incubated with trypsin. After trypsin inactivation the pelleted ganglia were triturated and resuspended in neuronal culture media with appropriate growth factors. The neuronal cell suspension was added to one empty somal reservoir and capillary action pulled the suspension across the channel into the opposite somal reservoir. Neurons readily attached to the poly-dl-ornithine and laminin substrate coated coverslip after one hour. Microscopic evaluation showed around 150-200 neuronal cell bodies in the somal channel and the axonal compartment contained no cells (Fig 1). Both of the somal reservoirs also contained considerable amounts of cell bodies that were unable to remain attached in the channel.

Neurons Seeded in the Somal Chamber Have Robust Outgrowth of Neurite Projections

In vitro cultures of dissociated embryonic rabbit dorsal root ganglia exhibited neurite outgrowth within one day of plating. Assessment of each somal compartment showed that healthy neuronal cell bodies remained attached to the substrate although a small percentage of cells detached and flowed into the somal reservoirs. Neurite outgrowth was visible within three

days from the neuronal cell body located in the somal compartment. Also, these neurites extended across the multiple microgrooves into the axonal compartment. By the ninth day post harvesting the axonal compartment showed an extensive network of neurites. After 12 days in culture the neuronal cell bodies located in the somal compartment and the neurites located in the axonal compartment were readily labeled with rabbit neurofilament specific antibody (Fig 2).

Microfluidic Chamber Compartmentalized Neuron Culture System Analysis of Axonal Transport

We used the microfluidic chamber system to study the axonal transport of Bovine herpesvirus type I Us9 mutants and rescued viruses. The microfluidic culture chamber system allowed for successful study of axonal transport. The system uses a fabricated PDMS (polydimethylsiloxane) polymer positioned on a glass coverslip (Fig 1). The chambers contain a microgroove length of 450 μm and a width of 10 μm which connects the somal and axonal compartments (Fig 1). The microgrooves provided a barrier that allowed fluidic isolation of neuronal cell bodies but allowed neurite outgrowth from the somal to the axonal compartment. The isolation of neuronal cell bodies in the somal compartment also created an environment where intact axons were the only connection between axonal compartment containing MDBK cells and axons. Fluidic isolation also created a hydrostatic pressure difference between each respective compartment that allowed for isolation of virus in either the somal or axonal compartment. Our data was analogous to PRV results with microfluidic chamber isolation of virus in respective compartments and neuron-to-cell spread of infection that is

dependent on intact axons (Liu 2008). The microfluidic chamber system allowed in vitro neuronal transport studies for BHV-1.

Infection of the Neuron Cell Body Can Occur Through Retrograde Infection of the Axon Termini in the Absence of BHV-1 Us9 Protein

We tested to determine if BHV-1 Us9 deleted, BHV-1 Us9 83-90 acidic domain deleted, and BHV-1 Us9 83-90 rescued viruses were capable of retrograde axonal transport in the microfluidic chamber system as demonstrated in vivo. Previous in vivo studies in calves and rabbits showed that both BHV-1 Us9 deleted, 83-90 acidic domain deleted, and 83-90 rescued viruses are capable of retrograde transport from the nasal and ocular epithelium post synaptic neurons to the presynaptic neurons of the trigeminal ganglia (TG) (Butchi 2007, Chowdhury manuscript in progress).

After two weeks the axonal compartment had dense neurite outgrowth in the microfluidic chambers. Before viral infection Madin-Darby bovine kidney cells (MDBK) were plated into the axonal compartments of the respective chambers. Retrograde infection of axon termini in the axonal compartment was performed 24 hours after addition of MDBK cells using a high multiplicity of infection (MOI) of each virus. With the exception of BHV-1 Us9 deleted, all viruses contained one copy of BAC and all recombinant strains expressed diffusible GFP. Thus, virus infection of neuronal cell bodies and MDBK cells in the somal and axonal compartments were detectable by live cell imaging of green fluorescence protein emission (Fig 3). After 12hours, green fluorescence was detected for all three viruses; BHV-1 Us9 deleted, BHV-1 Us9 83-90 acidic domain deleted, and BHV-1 Us9 83-90 rescued, in the somal compartments after infection of the axonal compartment (Fig 3). For all the three viruses tested, viral plaque assays

at 48 hours post infection ranged between $1-1.5 \times 10^8$ PFUs in the axonal compartment and $1-2 \times 10^3$ PFUs, in the somal compartments. All three viruses infected neuronal cell bodies in the somal compartment suggesting that each virus was capable of retrograde spread by entering the axon termini, traveling through the axons, and establishing infection in nerve cell bodies (Fig 3).

BHV-1 Us9 Deleted and BHV-1 Us9 83-90 Acidic Domain Deleted Viruses are Defective in Anterograde Axon Mediated Spread from the Neuron Cell Body to the Axon Termini

Next, we tested to determine if BHV-1 Us9 deleted and BHV-1 Us9 83-90 acidic domain deleted viruses were defective in anterograde transport and if BHV-1 Us9 83-90 rescued virus was capable of anterograde axonal transport in the microfluidic chamber system as demonstrated in vivo. To test the anterograde axonal transport property of the BHV-1 Us9 mutants and Us9 83-90 rescued viruses, a high MOI of virus was added to the neuron cell bodies in the somal compartments. MDBK cells were plated in the axonal compartment 24 hours prior to infection and the exact BHV-1 viruses were used as before in the retrograde experiment. At 24 hours post infection, live cell imaging showed that green fluorescence was only detected in the axonal compartments of the Us9 83-90 rescued virus but not in the Us9 deleted or Us9 83-90 deleted viruses (Fig 4,5,6). At 62 hours post infection, the final time point, green fluorescence was still only present in the axonal compartment of the Us9 rescued virus and not detectable in the axonal compartments of Us9 deleted and Us9 83-90 deleted viruses (Fig4,5,6). Viral plaque assay at 62hpi yielded very similar titers (4×10^6 - 9×10^6 PFUs) for the BHV-1 Us9 83-90 rescued, BHV-1 Us9 deleted and BHV-1 Us9 83-90 deleted in the somal

chambers. However, the plaque assay from the axonal chambers showed that there were no infectious particles in the axonal compartment for the BHV-1 Us9 deleted and BHV-1 Us9 83-90 deleted viruses. These results indicate that Us9 is required for anterograde spread from the nerve cell body to the axon termini.

CHAPTER 4 – Discussion

Previously, a method of establishing primary neuronal culture from fetal rabbits had not yet been published. Embryonic rabbits were used because among common laboratory animals, the rabbit is the only species susceptible to BHV-1, so the cultures were developed from neurons of the dorsal root ganglia (DRG) of rabbit embryos (Abril 2004, Rock 1982). Earlier studies showed that mice with a functioning immune system were able to prevent infection of BHV-1 due to a Th1 response and thus are not compatible for use in our system (Abril 2004). Earlier publications used mouse primary neuronal cultures for the microfluidic chamber system and we needed a system for rabbit dorsal root ganglia (Ch'ng 2006, Park 2006).

In this study, the results showed that embryonic rabbit dorsal root ganglia provided viable neuron cell bodies that survived in culture. Neuron cell bodies exhibited neurite outgrowth within one day of dissociation and within three days the axons crossed the microgrooves into the axonal compartment. Previous studies from rat superior cervical ganglia cultured in a microfluidic chamber showed similar results with robust outgrowth from the somal chamber and axons crossing the microgrooves into the axonal chamber (Liu 2008). While culturing ganglia, not every neuron cell body remained attached to the substrate coated coverslip due primarily to neurons dying, rough trituration, or poor handling techniques during media changes. Overall, the microfluidic chamber culture provided an appropriate system for growth of embryonic rabbit dorsal root ganglia.

Embryonic rabbit primary dorsal root ganglia in a microfluidic chamber culture system provided a suitable platform for studying *in vitro* anterograde and retrograde neuronal transport properties of BHV-1 viruses. Fabrication and assembly of the chamber has been previously described for various uses in neuroscience research (Park 2006, Liu 2008). The microfluidic chamber consists of two compartments, somal and axonal, connected by multiple microgrooves. In the culture system, dorsal root ganglia were plated into the somal compartment and growth of the axons extended from the nerve cell bodies and projected through the microgrooves and into the axonal compartment. Virus infection was utilized in the microfluidic chamber system by fluidically isolating virus to either respective compartment by hydrostatic pressures (Park 2006). The system was also useful for live cell imaging and indirect immunofluorescence assays.

In this study, we authenticated the microfluidic chamber culture system for analysis of neuronal transport of BHV-1. Previously, our laboratory used a rabbit and calf model for *in vivo* neuropathogenesis experiments and lacked a suitable *in vitro* system that could study complex neuronal viral transport properties of BHV-1. We observed that infection of the neuron cell body in the somal compartment can occur through retrograde infection of the axon termini located in the axonal compartment. Retrograde infection of BHV-1 necessitates essential glycoproteins; gB, gC, gD, gH, and gL because they are required for cell binding, fusion, and entry but does not require Us9 (Mettenleiter 1994, Ch'ng 2006). During retrograde infection viral particles enter axon termini following primary infection of nasal and ocular mucosal surfaces and are then transported to the neuron cell bodies in the trigeminal ganglia. The microfluidic chamber system allowed for fluidic isolation of virus to the axonal compartment

assuring that axon mediated transport was the only means of viral infection in the somal compartment (Park 2006, Liu 2008). Also, the results paralleled recent in vivo studies in calves and rabbits with BHV-1 Us9-deleted and Us9 83-90 acidic domain deleted viruses which indicated that Us9 is not required for retrograde transport of the virus from the peripheral sensory nerves to neuron cell bodies in trigeminal ganglia (Butchi 2007, Chowdhury manuscript in progress).

Interestingly, we found that BHV-1 Us9 deleted and BHV-1 Us9 83-90 acidic domain deleted viruses were defective in anterograde axonal mediated neuronal transport from the cell body into the axon termini. These results reiterate our previous in vivo studies in rabbits and calves with BHV-1 Us9-deleted and Us9 83-90 acidic domain deleted viruses such that Us9 is required for anterograde axonal transport of the virus from neuron cell bodies in trigeminal ganglia to sensory nerve endings in the nasal and ocular epithelium (Butchi 2007, Chowdhury manuscript in progress). Also, our results indicated that anterograde infection with BHV-1 Us9 83-90 rescued virus infected axon termini and MDBK cells of the axonal compartment indicating successful spread of virus through axons. These results concurred with our previous in vivo studies and also paralleled previous results showing that PRV Us9 null mutants were defective in anterograde spread of infection in the rat visual system, cultured neurons, and in vitro microfluidic chambers (Tomishima 2001, Lyman 2001, Liu 2008).

Our results agreed with PRV studies showing that mutation of two tyrosines and two serines within the acidic domain of Us9 terminated anterograde transneuronal spread of infection in the rat brain and hindered axonal sorting in dissociated primary neuronal cultures

(Brideau 2000, Lyman 2009). The BHV-1 Us9 83-90 acidic domain cluster contains key serine and tyrosine residues analogous to PRV (Brideau 2000, Lyman 2009). Together with our results, the fact that primary amino acid sequences and conserved domains of PRV and BHV-1 Us9 proteins are highly similar and BHV-1 Us9 homolog's functionally compensated for the loss of PRV Us9 in transneuronal spread this suggests that the acidic domain cluster must be required for anterograde neuronal spread in vivo and in our in vitro microfluidic system (Brideau, Lyman 2009, Chowdhury 2006, Chowdhury manuscript in progress).

In summary, we report that fetal rabbit dorsal root ganglia microfluidic chamber system provided a suitable platform for neuronal transport studies. Also, we concluded that BHV-1 Us9 protein is required for anterograde neuronal transport from the nerve cell body to the axon termini. Further studies will focus on the location of virus assembly within the neuron. The significance of BHV-1 Us9 protein acidic domain deletions also require further study to determine their relevance in targeting structural proteins to the axon and subsequent axonal anterograde transport properties in the in vitro microfluidic compartmentalized chamber system.

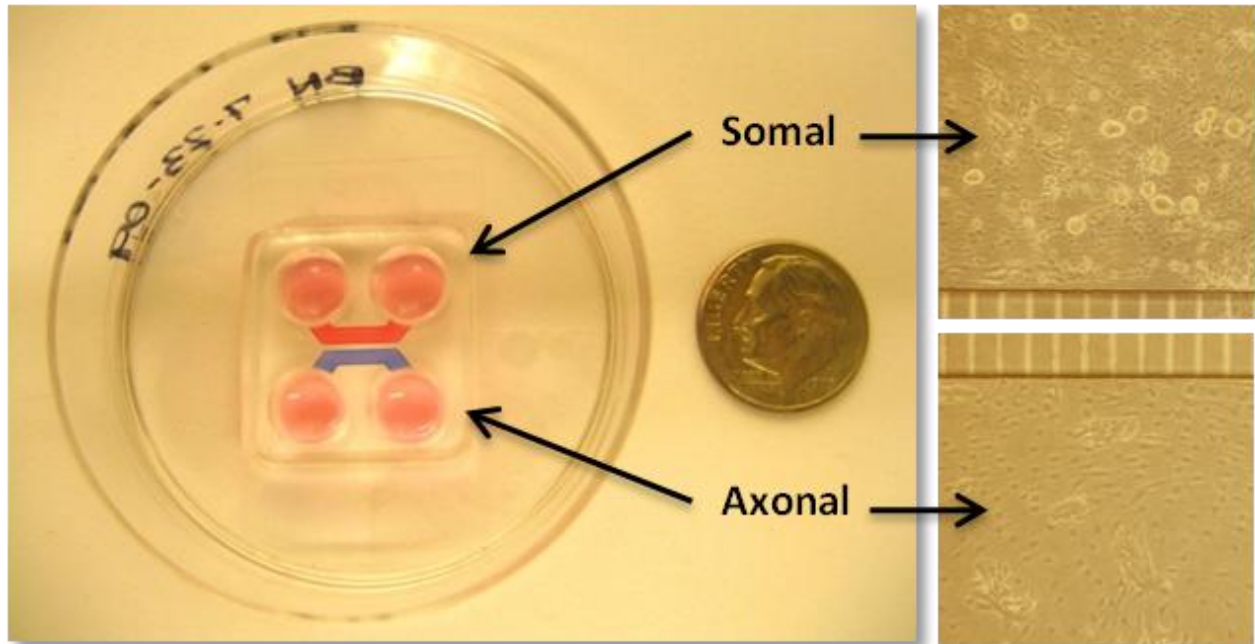


Figure 1. Fundamentals of the microfluidic chamber culture system.

The chamber system uses a fabricated PDMS (polydimethylsiloxane) polymer positioned on a glass coverslip. The microfluidic chamber is comprised of two compartments, the somal and axonal, connected by microgrooves (10 μm in width and 450 μm in length). The microgrooves provide a barrier and a passageway that allows for fluidic isolation of the neuron cell bodies in the somal compartment and growth of axons into the axonal compartment. These images are of phase contrast microscopy of rabbit DRG neurons after two weeks in the microfluidic chamber.

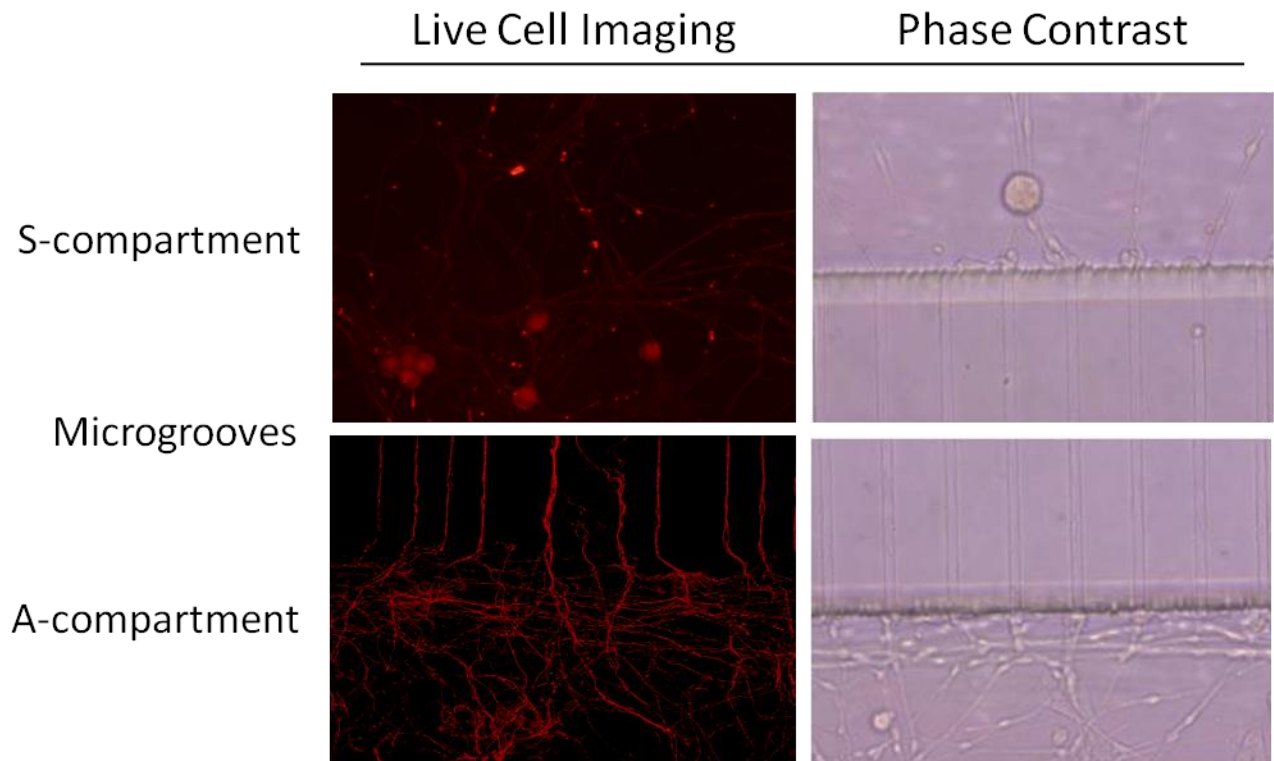


Figure 2. Rabbit dorsal root ganglia provide viable neurons for in vitro use.

Embryonic rabbit DRG neurons exhibited neurite outgrowth within one day of plating. After 12 days in culture, DRG neurons located in the somal compartment and the neurites located in the axonal compartment were labeled with primary rabbit neurofilament specific antibody and secondary Alexa fluor 594 Donkey anti Rabbit antibody and detected by indirect immunofluorescence assays.

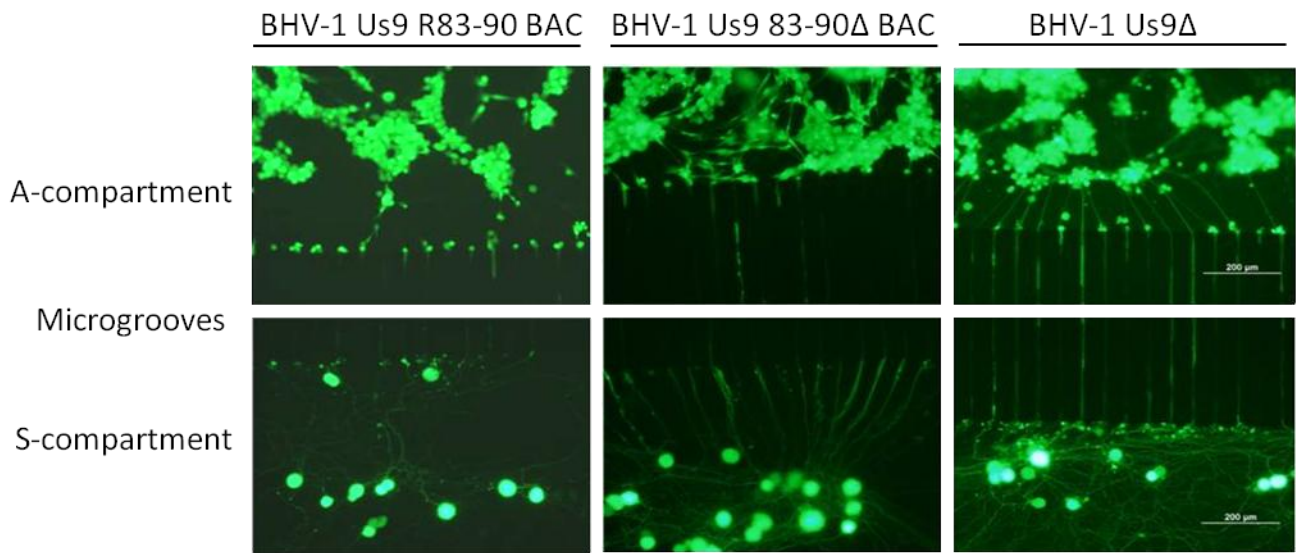


Figure 3. Infection of the neuron cell body can occur through retrograde infection of the axon termini in the absence of BHV-1 Us9 protein.

Neurites in the axonal compartment were infected at high MOI with each respective virus; BHV-1 Us9 deleted, BHV-1 Us9 83-90 acidic domain deleted, and BHV-1 Us9 83-90 rescued (all viruses express GFP). Detection of GFP was done by live cell imaging starting at 12 hours post infection of the somal compartment, microgrooves, and axonal compartment of each microfluidic chamber.

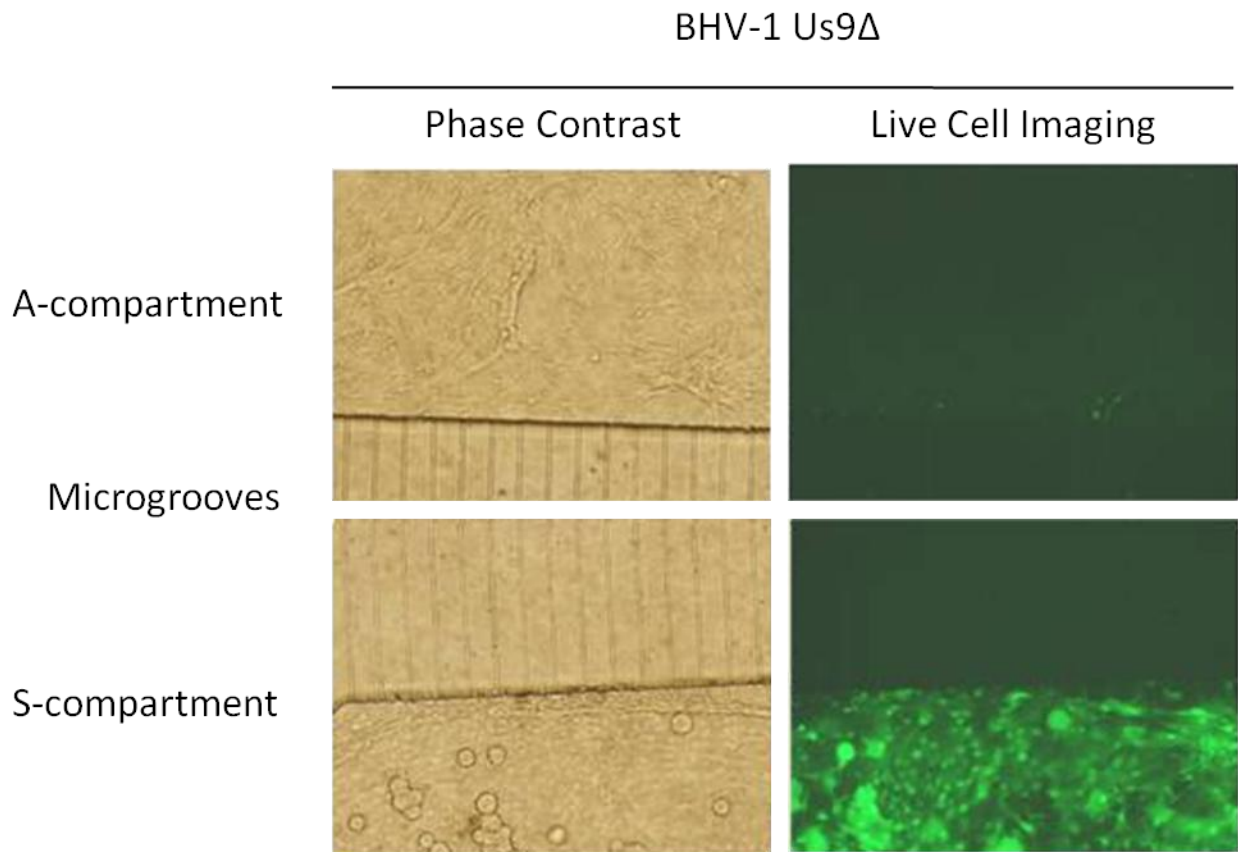


Figure 4. BHV-1 Us9 deleted virus is defective in anterograde neuronal spread from the neuron cell body into the axon.

Neuron cell bodies in the somal compartment were infected at high MOI with BHV-1 Us9 deleted virus (expressing GFP). Detection of GFP was done by live cell imaging at 62 hours post infection of the somal compartment, microgrooves, and axonal compartment of each microfluidic chamber.

BHV-1 Us9 83-90 Δ BAC

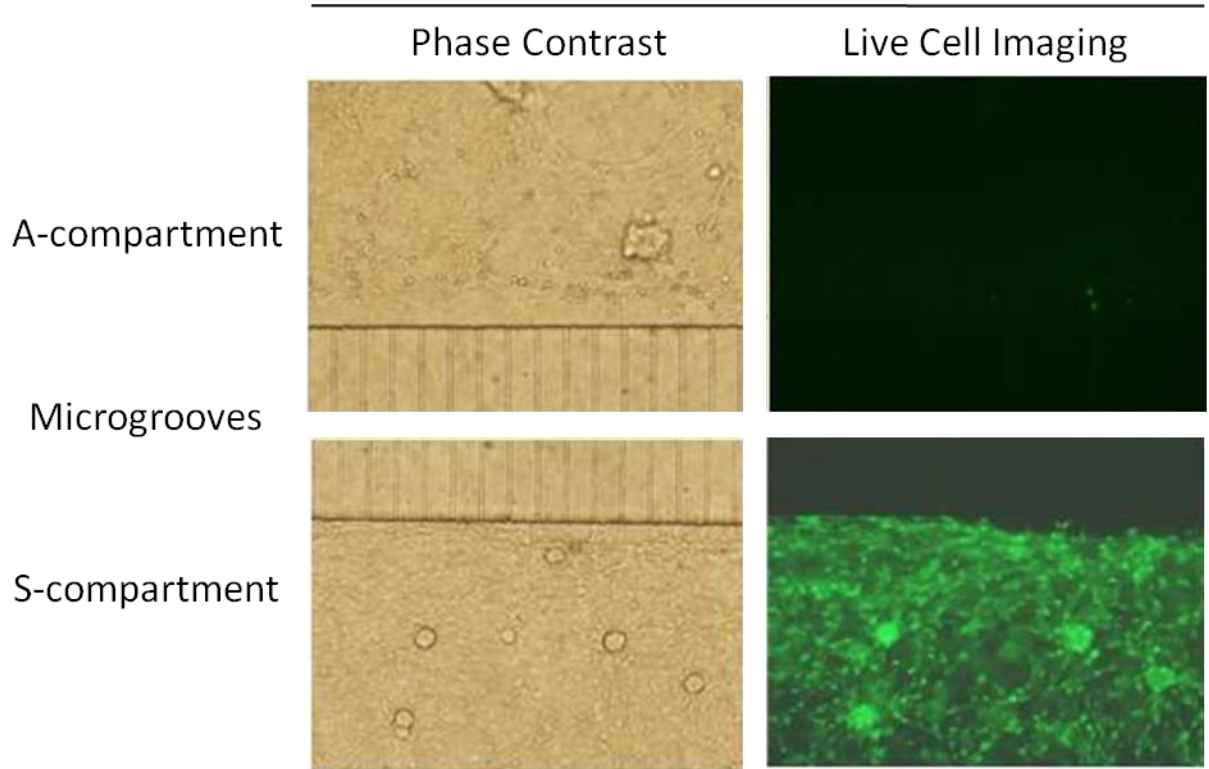


Figure 5. BHV-1 Us9 83-90 Δ BAC virus is defective in anterograde neuronal spread from the neuron cell body into the axon.

Neuron cell bodies in the somal compartment were infected at high MOI with BHV-1 Us9 acidic domain deleted virus (expressing GFP). Detection of GFP was done by live cell imaging at 62 hours post infection of the somal compartment, microgrooves, and axonal compartment of each microfluidic chamber.

BHV-1 Us9 R83-90 BAC

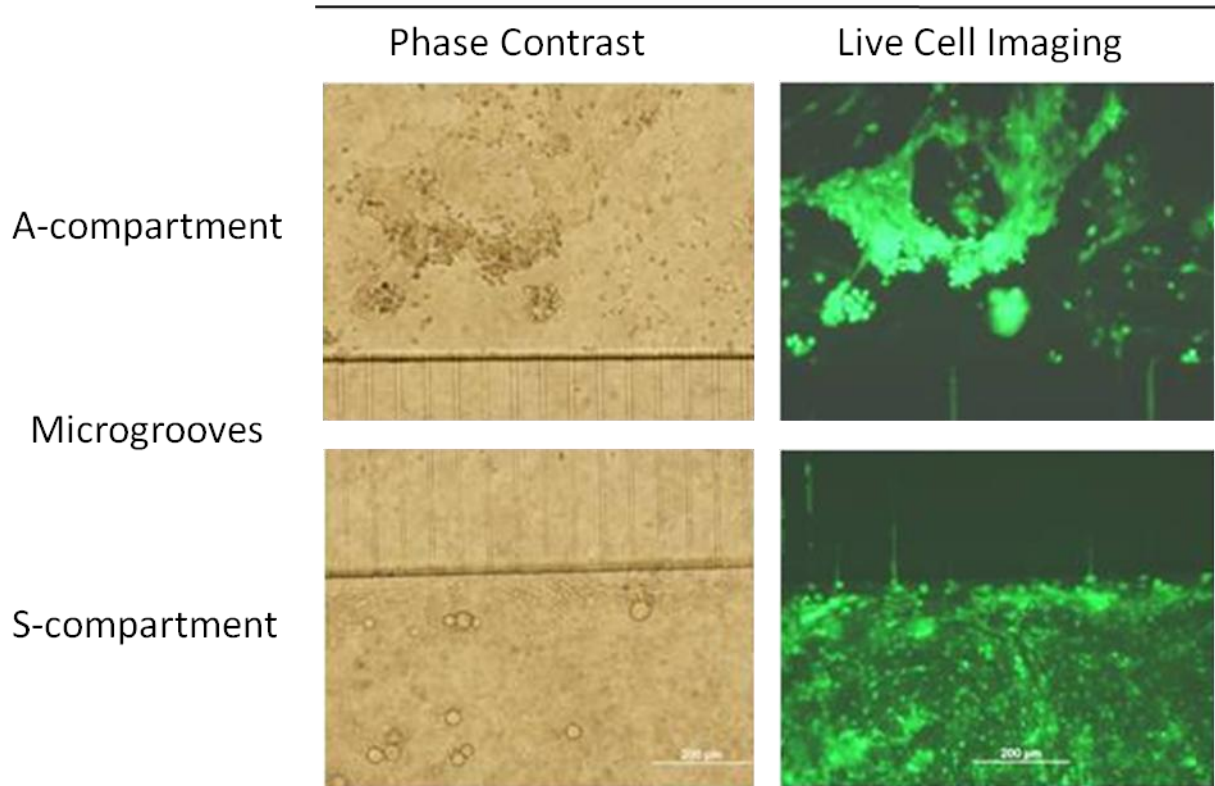


Figure 6. BHV-1 Us9 R83-90 Δ BAC virus successfully infects axon termini after anterograde infection of the neuron cell body.

Neuron cell bodies in the somal compartment were infected at high MOI with BHV-1 Us9 83-90 rescued virus (expressing GFP). Detection of GFP was done by live cell imaging at 62 hours post infection of the somal compartment, microgrooves, and axonal compartment of each microfluidic chamber.

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