Video Article

# Adult Mouse DRG Explant and Dissociated Cell Models to Investigate Neuroplasticity and Responses to Environmental Insults Including Viral Infection

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## Abstract

This protocol describes an *ex vivo* model of mouse-derived dorsal root ganglia (DRG) explant and *in vitro* DRG-derived co-culture of dissociated sensory neurons and glial satellite cells. These are useful and versatile models to investigate a variety of biological responses associated with physiological and pathological conditions of the peripheral nervous system (PNS) ranging from neuron-glial interaction, neuroplasticity, neuroinflammation, and viral infection. The usage of DRG explant is scientifically advantageous compared to simplistic single cells models for multiple reasons. For instance, as an organotypic culture, the DRG explant allows *ex vivo* transfer of an entire neuronal network including the extracellular microenvironment that play a significant role in all the neuronal and glial functions. Further, DRG explants can also be maintained *ex vivo* for several days and the culture conditions can be perturbed as desired. In addition, the harvested DRG can be further dissociated into an *in vitro* co-culture of primary sensory neurons and satellite glial cells to investigate neuronal-glial interaction, neuritogenesis, axonal cone interaction with the extracellular microenvironment, and more general, any aspect associated with the neuronal metabolism. Therefore, the DRG-explant system offers a great deal of flexibility to study a wide array of events related to biological, physiological, and pathological conditions in a cost-effective manner.

## Video Link

The video component of this article can be found at https://www.jove.com/video/56757/

## Introduction

In this manuscript, we report a method to obtain an organotypic *ex vivo* model of a mouse derived DRG model system as a preserved tissue-like microenvironment to investigate a wide range of biological responses to PNS insults ranging from neuron-glial interaction, neuroplasticity, inflammatory markers, to viral infection. In addition, we further developed a protocol to create a primary co-culture of DRG-derived single sensory neurons and satellite cells.

The DRG are satellite gray-matter-units located outside the central nervous system (CNS) along the dorsal spinal roots of spinal nerves. The DRG, located in proximity of intervertebral foramina, house pseudounipolar sensory neurons and satellite glial cells. The pseudounipolar neurons feature a single neurite that splits into a peripheral process carrying somatic and visceral inputs from peripheral targets to the cell body, and a central process that submits sensory information from the cell body into the CNS. A connective capsule defines and isolates this peripheral cluster of neurons and glial cells from the CNS. No postnatal cell migration to or from the DRG has ever been described and a local stem cell niche is responsible for neurogenic events occurring throughout life[1]. Therefore, this model is particularly suitable to study adult neurogenesis, axonogenesis, response to traumatic lesion, and cell death[2,3,4,5,6,7,8,9] .

In the field of neuroregeneration, the DRG harvested from *in vivo* and explanted *in vitro* reproduces axonotmesis, an injury condition in which axons are fully severed and the neuronal cell body is disconnected from the innervated target[10,11]. It is well known that peripheral nerve injury can cause decreased and increased gene expression in the DRG and many of these changes are a result of regenerative processes but many may also be a result of immune response or another response from non-neuronal cells. By using an *ex vivo* system of isolated DRG, some of this complexity is removed and mechanistic pathways can be more easily investigated.

Besides its central role in conveying sensory inputs to the CNS, the abundance of receptors for many neurotransmitters including GABA[12,13,14,15] at the level of the neuronal soma as well as evidence of interneuronal cross-excitation may suggest that DRG are sophisticated preliminary integrators of sensory inputs[16,17]. These new findings confer to the DRG explant the characteristics of a mini-neuronal network system similar to other "mini-brain" models, which are nervous-tissue-specific organoids used for broader experimental fields of investigation and therapeutic

 

approach to neurological diseases[18,19]. These evidences together with the fact that the DRG is a discrete and well-defined cluster of neuronal tissue surrounded by a connective capsule, make it a suitable organ for *ex vivo* transplantation.

Culturing mouse DRG presents an attractive multicellular option to model human pathophysiologies due to structural and genetic similarities between the species. Additionally, a large repository of transgenic mouse strains is highly conducive to future mechanistic studies. Neurite extension both during development and after injury requires mechanical interactions between growth cone and substrate[20,21]. Nano- and micro-patterned substrates have been used as tools to direct neurite outgrowth and demonstrate their capacity to respond to topographical features in their microenvironments. Neurons have been shown to survive, adhere, migrate, and orient their axons to navigate surface features such as grooves in substrates[22,23]. However, these studies have typically utilized cultured cell lines and it is difficult to predict how primary neuronal cells will respond to well-defined, physical cues *in vivo* or *ex vivo*.

The *ex vivo* explant model of mouse DRG used for this proposal mimics the real cell-cell interaction and biochemical cues surrounding growing axons. Among many different experimental paradigms ranging from axonal regeneration, neurosphere production, to neuroinflammation, the DRG explant model continues to serve as a valuable tool to investigate the viral infection and latency aspect within sensory ganglia[24,25,26,27].

The nervous system (NS) in general is target for viral infections[28,29,30]. Most viruses infect epithelial and endothelial cell surfaces and make their way from the surface tissue to the NS via peripheral nerve sensory and motor fibers. In particular, the herpes simplex virus type 1 (HSV-1) after an initial infection in epithelial cells establishes a life-long latency in the sensory ganglia preferably, the DRG of the PNS[31,32]. HSV-1 neuroptropic capability of infecting the PNS ultimately leads to neurological diseases[33].

## Protocol

All the procedures including the use of the animals have been approved by the institutional review board-approved protocols (IACUC-Midwestern University).

## 1. Harvesting DRG from Mouse Embryos

1. **Euthanize the adult mice by asphyxiation method ($CO_2$) followed by decapitation. Immediately proceed to surgically remove the vertebral column.**
    1. Expose the vertebral column by cutting down the skin layer dorsally using fine scissors. Isolate the vertebral column by cutting through the ribs on either side of the column and through the sacrum separating the vertebral column from the rest of the animal.
    2. Mount the vertebral column (ventral side up) onto a surgical mat using needles/pins.
2. Using fine scissors make a double cut on both sides of the vertebral bodies to expose the ventral side of the vertebral canal.
3. Under a surgical microscope (magnification set to 4X), use scissors to gently move the spinal cord on the side to expose the contralateral dorsal spinal roots and to locate the DRG, along the dorsal roots of the peripheral nerves. Each ganglion is partially hidden inside the intervertebral foramina.
4. To harvest the DRG, pinch the dorsal root (between the spinal cord and the DRG) with one forceps and gently pull out the DRG from the intervertebral foramen.
5. Place the second forceps on the spinal nerve peripheral to the DRG and pull the DRG together with the spinal nerve and spinal root.
6. Place the collected DRG in a 35 mm Petri dish containing 3 mL of ice-cold serum free media (SFM)[34].
7. Transfer each individual DRG in a dry glass Petri dish and, under a surgical microscope, clean and trim off excess fibers and connective tissue still attached to the DRG using a blade. The DRG is easily identifiable as a bulgy transparent structure along the white spinal nerve/root. Blood vessels often are found surrounding the DRG.
8. Place the cleaned DRG in a new Petri dish containing ice-cold SFM media.
9. Dilute the gelatinous protein mixture (see **Table of Materials**) in ice-cold SFM (1:1).
10. Plate the DRG *ex vivo* in 12-well plates pre-coated with 10/20 µL of gelatinous protein mixture and set them inside the incubator at 37 °C and 5% $CO_2$ for 30-60 min.
11. Gently add 1.5-2 mL of SFM to the culture system to cover the entire explant and maintain the explants at culturing conditions (37 °C and 5% $CO_2$).
    NOTE: This is a critical step because the DRG is anchored to the glass dishes only by the polymerized gelatinous protein mixture. Time of polymerization and pipetting skills are critical to avoid floating.
12. Change the medium of growing DRG every 72 h and let the DRG grow for as long as needed.

## 2. Isolating Single Cell Neurons from DRG

1. Place all the DRG collected in a 1.5 mL sterile tube with 1.2 mL of F12 media containing 1.25 mg/mL of collagenase IV and incubate it at 37 °C and 5% $CO_2$ for 45 min. Repeat this step for another 45 min after the first incubation.
2. Treat explants with 2 mL of F12 media containing trypsin (0.025%) for 30 min immediately after the collagenase IV treatment at 37 °C and 5% $CO_2$.
3. Incubate with 2 mL of F12 media containing fetal bovine serum (FBS; 33%) at 37 °C and 5% $CO_2$ for 15 min.
4. Wash explants three times with 2 mL of F12 media and proceed to mechanically dissociate them with a glass pipette until the media turns cloudy.
    NOTE: This procedure involves collection of clean/trimmed DRG from the animal as explained in the prior section. When performing mechanical dissociation of explants, be gentle and do not use excessive force since it can lead to spillage and loss of working material.
5. Filter the dissociated cell culture through a 0.22 µm filter to remove any impurities and excess connective tissue. Centrifuge the filtered cell lysate at (2,500 x g) for 2 min.

6. Remove the supernatant and resuspend the cell pellet in 500 µL of neurobasal media containing supplement for neuronal culture (1x), antibiotic mixture (1x), L-glutamate (0.5 mM), and nerve growth factor (5 µg/mL) (see **Table of Materials**).
7. Plate the dissociated cells onto laminin-coated cover slides (50 µg/mL; see **Table of Materials**) at a preferred cell density. Determine the cell density using a cell counter.
   NOTE: We plated cells at 25,000 cells/cover slide. This technique allows dissociation of both neurons and glial satellite cells. The glial component co-cultured with the pseudounipolar neurons plays a critical role for neuronal survival.

## 3. HSV-1 Infection of DRG Explants and DRG-derived Dissociated Cells

NOTE: This work was done by strictly following the biosafety level-2 (BSL-2) requirements to which we have a fully equipped lab that is approved by the Midwestern University biosafety committee. A KOS strain of HSV-1 was used in this study. Please take appropriate measurements and safety precautions as per local institutions guidelines if working with virus strains.

1. Determine and prepare the virus in the correct dilutions in SFM media to infect the model. The virus used in this study was KOS strain of HSV-1. When working with DRG-derived dissociated cells, use 1 unit of multiplicity of infection (MOI) for infection, which means the number of virus equal the number of cells.
2. If infecting DRG explants, use the number of virions (*e.g.*, 10,000 virions) because an exact number of cells in an explant cannot be determined.
3. Place the explant/cells to be infected with virus in a sterile cell plate or tube containing a mixture of SFM media and virus.
   NOTE: We used 25,000 virions for a cover slide containing 25,000 cells (1 MOI). To infect an explant, we use 10,000 virions.
4. Place the cell plate or tube at 37 °C for infection to take place; time of viral exposure may vary depending on viral infectivity.
   NOTE: Viral entry was confirmed by using ortho-nitrophenyl-β-galactoside (ONPG) and 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) assays[26].

## 4. Immunofluorescence

1. Fix the explants and single cell samples in 4% formalin prepared in phosphate buffered saline (PBS). Wash samples 3 times for 10 min each in PBS.
2. Incubate the samples with desired primary antibodies (anti-β-tubulin, anti-peripherin, anti-heparan sulfate (HS), and/or anti-glycoprotein D (gD) antibody; see **Table of Materials** for dilutions) diluted in PBS buffer with 0.3% Triton-X (PBST) and 10% normal goat serum. Store samples at 4 °C overnight.
3. Wash the samples 3 times for 10 min each with PBS. Incubate samples at room temperature for 1 h in the appropriate secondary antibody (488 and/or Cy3; see **Table of Materials** for dilutions) diluted in PBS.
4. Wash the samples 3 times for 10 min each with PBS. Incubate the samples with Hoechst dye (1.5 µM) in PBS for 20 min prior to mounting step.
5. Mount the samples on glass slides using a fluorescence mounting medium and coverslip.

## Representative Results

Multiple aspects of neuroplasticity and neuron-environment interaction can be investigated using DRG and a single dissociated cell culture model. We began the studies by isolating a DRG explant and DRG-derived dissociated cells as schematically represented in **Figure 1**. Both tissue and single cells models can be analyzed by using a variety of molecular techniques such as immunofluorescence, Western blot, genomic assays, and other analytical techniques depending on the nature of experimental design and aims. First, we used our DRG explant model to investigate the effect of biochemical cues on axonal growth (**Figure 2**). In double or triple immunofluorescence, the differential expression of peripherin (**Figure 2A**, B; red) and β-tubulin (**Figure 2A**, B; green) were confirmed both within the neuronal cell bodies and the peripheral neurite sprouts. Cell nuclei were identified with Hoechst stain (**Figure 2A**; blue). The expression of the above-mentioned markers was further analyzed in the entire DRG explant after fixation in 4% paraformaldehyde and microtome-sectioning (**Figure 3A**). Our results confirmed that peripherin and β-tubulin are selectively expressed in two subpopulations of ganglionic neurons with peripherin being expressed in the "small, light" and β-tubulin in the "large, dark" subpopulations of neurons, respectively. The neurites immunolabeled with either marker emerging from the sensory neurons were found coursing through the entire ganglion before exiting into the periphery. Hoechst stain mostly identified the position of the glial satellite nuclei within the ganglion. As indicated in **Figure 3B**, the neuron-satellite cell interaction can be better visualized after the full cell dissociation technique. Blue-stained satellite cell nuclei were seen surrounding a β-tubulin-positive sensory neuronal soma and its extensive neurites.

We further utilized the DRG-derived dissociated cells to investigate HSV-1 infection and associated inflammatory response within the sensory neurons (**Figure 4**). After only 1 h of post infection (p.i.), HSV-1 entry was detected by using anti-HSV-1 gD antibody both in glial cells (**Figure 4A**) and DRG neurons (**Figure 4B**). In addition, HS staining was further carried out since HS provides a docking sites for virus attachment or binding to host cell. HS has been known as a critical component of the multi-functional extracellular matrix (ECM) and extensively documented to play a major role in cell adhesion, cell-to-cell signaling, and ECM remodeling during wound healing, embryonic development, cancer invasion, fibrosis, and in neuroinflammation[35,36]. Interestingly, we observed HS staining in the ECM suggesting its potential role in the microenvironment that allows for axonal growth (**Figure 4C**). Finally, for β-tubulin staining we used as a positive marker for the neurites to confirm the integrity of the neurons.

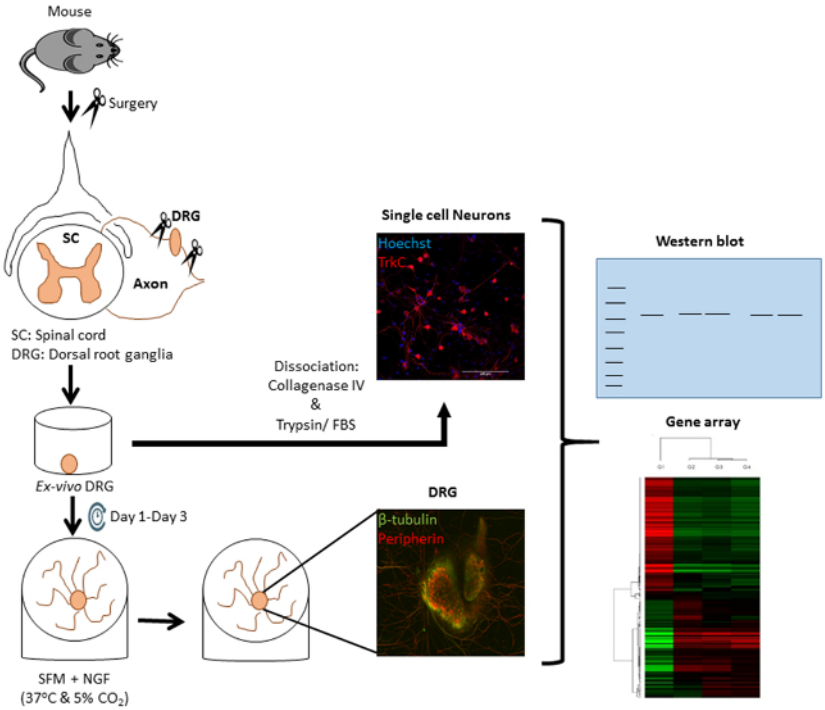


**Figure 1. Schematic representation of the experimental DRG and single cell culture models.** DRG were isolated from Adult NIH/SWISS mice, cleaned from excessive capsular connective tissue, and either explanted *ex vivo* as a whole explant or dissociated into primary co-cultures of sensory neurons and satellite cells. Both models can be used for further analyses with multiple analytical techniques. Please click here to view a larger version of this figure.

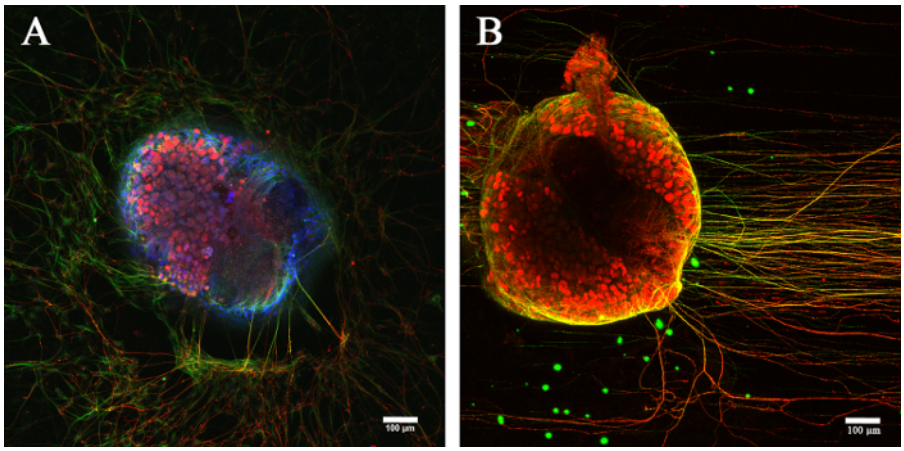


**Figure 2. Development of DRG organotypic culture as a model system to investigate axonal growth.** DRG grown for 6 days *ex vivo* were used to investigate axonal sprouting from the explant. We show by immunofluorescence how different biochemical conditions can affect the distribution of fiber sprouts: growing randomly and disorganized ( A) compared to a more linear and organized fashion ( B). The explants are labeled with anti-β-Tubulin (green) and anti-peripherin (red). Cell nuclei are stained with Hoechst stain (blue). Scale bars = 100 µm. Please click here to view a larger version of this figure.

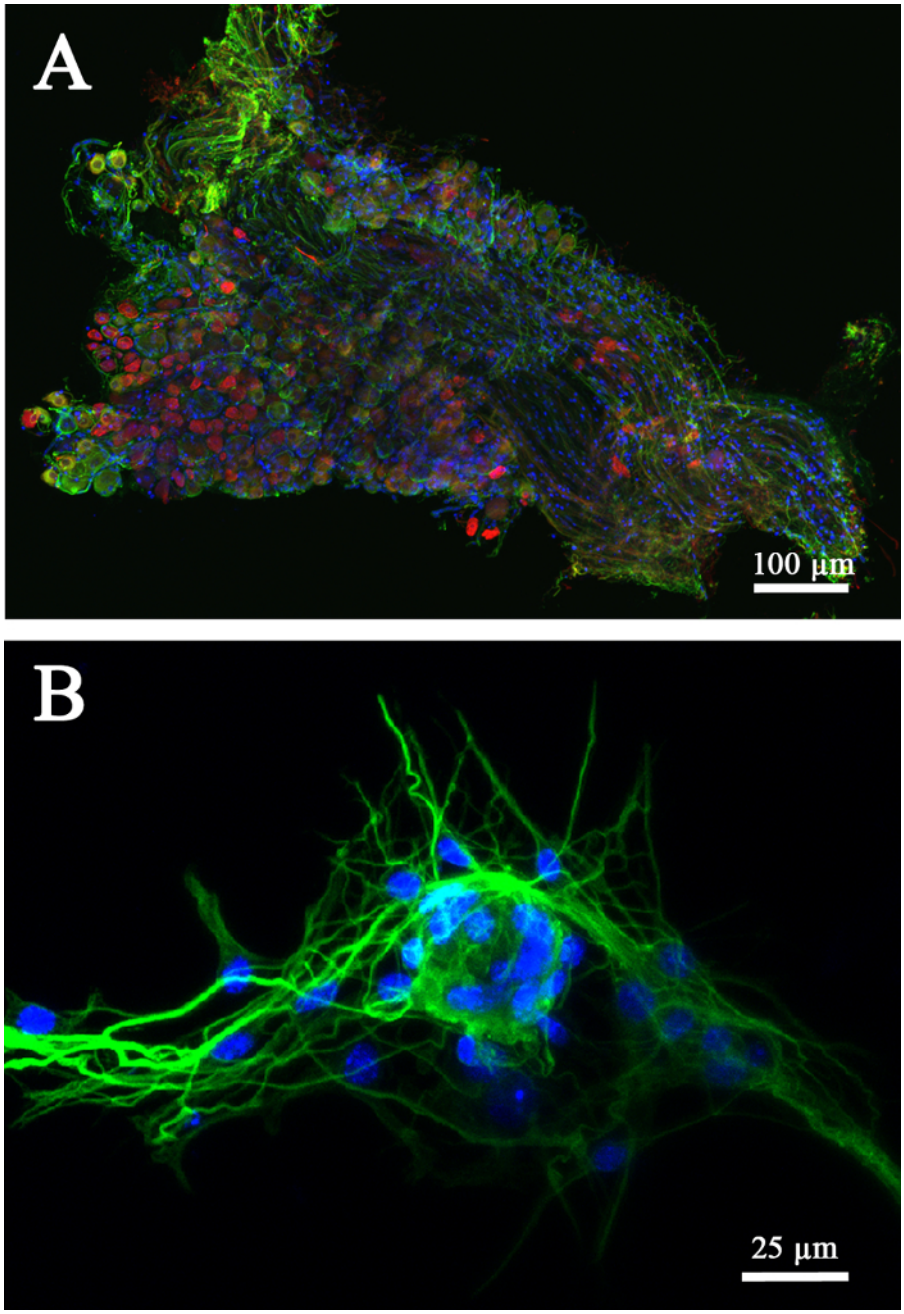


**Figure 3. Molecular characterization of the isolated neuronal culture model.** Sections of the whole DRG were immunolabeled with antibodies against peripherin and β-tubulin showing selective expression of the two markers in two subpopulations of ganglionic neurons. Peripherin (red) is expressed in the "small, light" neurons and β-tubulin (green) is expressed in the "large, dark" neurons. Hoechst stain mostly identified the position of the glial satellite nuclei within the ganglion ( A). The neuron-satellite cell interaction can be better visualized after full cell dissociation technique ( B). Here we show a β-tubulin-positive sensory neuronal body and its neurites surrounded by satellite glial cells (Hoechst: nuclei, blue). Scale bars, A = 100 µm; B = 25 µm. Please click here to view a larger version of this figure.

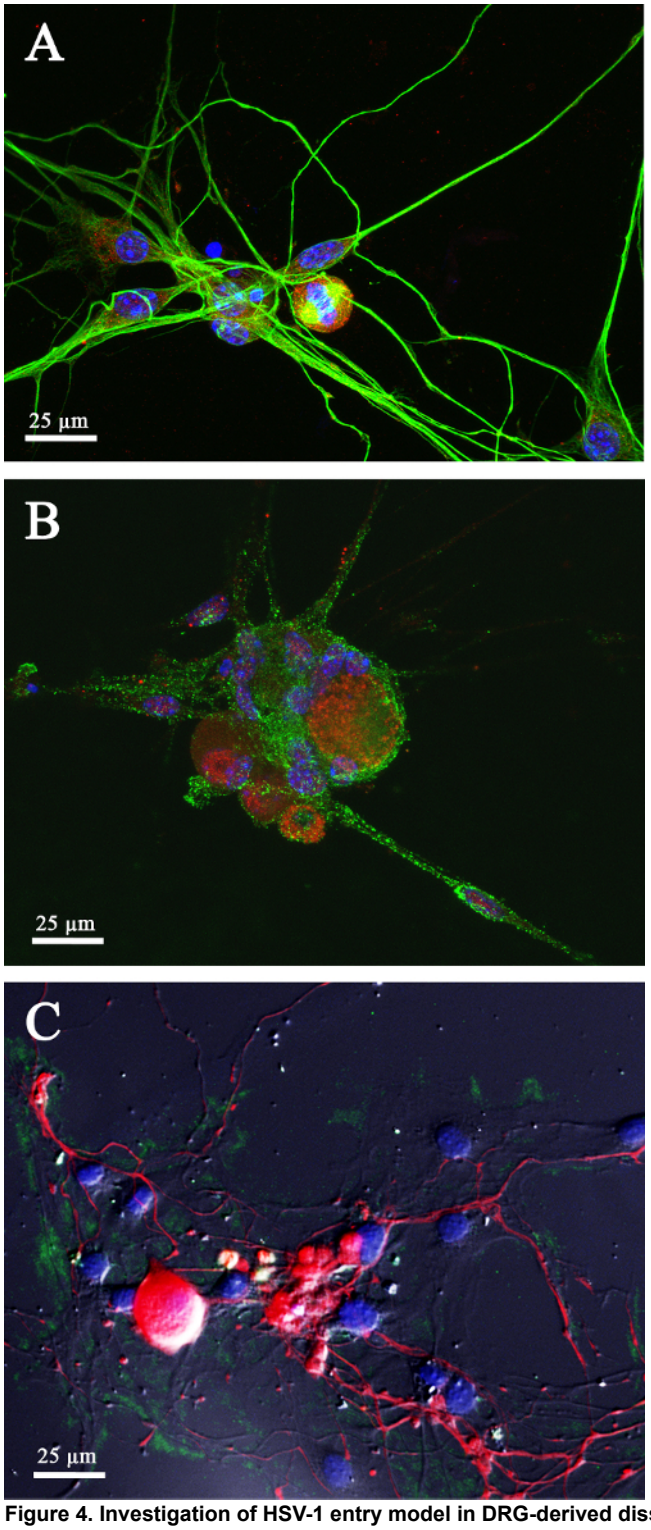


**Figure 4. Investigation of HSV-1 entry model in DRG-derived dissociated cells.** HSV-1 infection of dissociated satellite cells depicted along β-tubulin-positive neurites (green) is revealed using an anti-viral gD antibody (red). Satellite glial cells nuclei are stained blue with Hoechst ( A). The same antibody reveals viral entry in dissociated DRG neurons ( B). Cells are co-labeled with an antibody against heparan sulfate (HS, green) expressed on the cell membrane. HS is also a component of the extracellular matrix (green) and plays an important role for axonal growth ( C). Neurons are labeled with peripherin (red) while Hoechst blue is used to identify satellite cell's nuclei. Scale bars = 25 µm. Please click here to view a larger version of this figure.

## Discussion

The *ex vivo* DRG model is extremely useful to investigate a wide spectrum of events such as neuron-glia interaction as well as the effect of the microenvironment on both neuronal and glial metabolism[37]. Further, the DRG-model could be used as a cost-effective tool to address relevant questions regarding pathogenic mechanism and associated markers by developing ex *vivo* systems for acute chronic and latent phase of infection or in a given disease. In addition, a screening library of small molecules in DRG explants could exploit this explant model for drug development. *In vitro* models, especially single-cell systems, are often too simplistic to correlate to physiologically relevant events; while *in vivo* models are challenging to precisely manipulate, partially due to the immune response to injury, glial scarring, and the complexity of the surrounding ECM along with the financial expense. In contrast, the DRG explant is an organotypic culture that allows *ex vivo* transfer of an entire organ with the complexity of a neuronal network, including the extracellular microenvironment that plays a significant role in all the neuronal and glial functions. However, the generation of an *ex vivo* model can only resemble but not fully reciprocate all the conditions found *in vivo*. Two important limitations to keep in mind when using this model are the timing and the absence of systemic feedbacks, which can only truly be represented *in vivo*. The limited time that the DRG can be maintained vital *ex vivo* makes this model more suitable for investigation of acute conditions and thus not a very reliable model for chronic conditions. Similarly, the *ex vivo* model is not ideal for studies that may be effected by the absence of responses from other organs or systems (*e.g.*, the immune system).

We were able to successfully isolate the explant *ex vivo* DRG from both adult and embryo mice and rats and use this model to investigate HSV-1 infection as well as multiple other aspects of neuroplasticity and its effects on translational medicine. In order to allow sensory fibers to grow outside the explant, the connective surrounding capsule was partially removed[1].

To be considered, the gelatinous protein mixture extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcom isrich in ECM proteins such as laminin, collagen IV, heparin sulfate proteoglycans (HSPG), and a number of growth factors[38]. In this protocol, the gelatinous protein mixture is diluted 1:1 with culture medium and allowed to polymerize for 30-60 min at 37 °C before adding the final volume of medium. The gelatinous protein mixture polymerizes at room temperature; therefore, it is suggested to refrigerate the mixture until explants are plated and to use cold pipette tips to distribute 10-20 µL drops onto the 12-well dishes. Timing is critical for the final efficiency of the culture procedure and may vary depending on the type (and manufacture) of the gelatinous protein mixture used. If the gelatinous protein mixture is not fully polymerized or it is already solidified, the explant can float or dry out leading to unsuccessful growth. We encourage investigators to empirically determine the appropriate temperature, time, and gelatinous protein mixture dilution for a successful model. We reached a 55-70% efficiency of successful growth of DRG explants maintained *ex vivo* for up to 7-10 days. The explant can be further dissociated into DRG-derived primary sensory neurons and satellite cells co-culture.

## Disclosures

The authors have nothing to disclosure.

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