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Methods and Applications of Campenot Trichamber Neuronal Cultures for the Study of Neuroinvasive Viruses

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Abstract

The development of compartmentalized neuron culture systems has been invaluable in the study of neuroinvasive viruses, including the alpha herpesviruses Herpes Simplex Virus 1 (HSV-1) and Pseudorabies Virus (PRV). This chapter provides updated protocols for assembling and culturing rodent embryonic superior cervical ganglion (SCG) and dorsal root ganglion (DRG) neurons in Campenot trichamber cultures. In addition, we provide several illustrative examples of the types of experiments that are enabled by Campenot cultures: 1. Using fluorescence microscopy to investigate axonal outgrowth/extension through the chambers, and alpha herpesvirus infection, intracellular trafficking, and cell-cell spread via axons. 2. Using correlative fluorescence microscopy and cryo electron tomography to investigate the ultrastructure of virus particles trafficking in axons.

Key words Campenot, neurons, superior cervical ganglia, dorsal root ganglia, virus, alphaherpesvirus, herpes simplex virus, pseudorabies virus, fluorescence microscopy, cryo electron tomography

1 Introduction

Neuroinvasive viruses are capable of infecting both the peripheral and central nervous systems. Natural or nosocomial neuroinvasive virus infections are often acutely severe, causing a variety of neuropathies, demyelinating disease, meningitis, and encephalitis [1, 2]. It is also clear that some viruses, like the alpha herpesviruses (e.g. herpes simplex viruses, and varicella-zoster virus) can also spread to the central nervous system asymptomatically or sub-clinically, where they may cause chronic or recurrent

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neuroinflammation and trigger the development of neurodegenerative diseases like Alzheimer's [3]. While the spread of these viruses within the nervous system is not entirely understood, researchers have taken advantage of their neuroinvasive properties as neuroanatomical circuit tracing tools [2, 4, 5].

Culturing peripheral neurons *in vitro* is an important aspect of studying neuroinvasive viral infections. Two well-established culture systems frequently implemented for viral research are embryonic rodent Superior Cervical Ganglia (SCG) and Dorsal Root Ganglia (DRG). These ganglia innervate the head, or much of the rest of the body, respectively. SCGs are part of the sympathetic nervous system, innervating areas such as the eyes, pineal gland, salivary glands, and some thoracic targets, including the heart. The SCG was one of the first model systems to study neurotransmission [6]. SCG neurons are relatively homogenous in culture (compared to primary CNS cultures or neurons differentiated *in vitro* from stem cells), and while they are mostly noradrenergic *in vivo*, they often undergo a phenotypic change and become cholinergic *in vitro* [7]. SCG neurons are strictly dependent on nerve growth factor (NGF) trophic support, but do not require co-culture with support cells, and can be maintained in culture for weeks. DRGs are part of the sensory system, and consist of many subpopulations of neurons, such as proprioceptors, mechanoreceptors, thermoreceptors, and nociceptors. Mammalian DRG neurons are typically pseudounipolar *in vivo*, with one axonal process that innervates peripheral tissues, and another that conveys sensory information to the central nervous system. However, DRG neurons *in vitro* often exhibit variable numbers of axons, and it is unclear whether these axons maintain central vs. peripheral specializations. Different subpopulations of DRG

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neurons can be selected based on which neurotrophic factors are provided. DRG neurons do not require co-culture with support cells; however, it can be difficult to eliminate non-neuronal cells (such as Schwann cells) in DRG cultures [8, 9].

Studying the molecular and cell biology of virus infection in neurons has been greatly enhanced with novel live-cell imaging methods and fluorescent protein fusions. Recombinant viruses containing fluorescent protein fusions to viral structural proteins have been used to address viral entry, intracellular transport, and egress in neurons (e.g. [10-14]). However, a major difficulty is that the virus inoculum used to initiate the infection remains in typical dissociated neuron cultures, even for many hours post-infection. In strongly-adherent non-neuronal cell lines, depending on the virus, excess inoculum that has not yet entered cells can be reduced or eliminated by briefly treating cells with an acidic buffer, neutralizing antibodies, or extensive washes/medium changes. However, neuronal cultures typically do not tolerate such perturbation to remove or neutralize viral inoculum. When tracking individual fluorescently-labeled virus particles, it can be difficult to distinguish particles in the inoculum from progeny particles produced during infection. Given the very long distances virus particles may need to traffic in axons, progeny particles may be exiting while, simultaneously, inoculum particles are still entering infected cells [15]. In the alpha herpesvirus literature, this problem has led to great confusion over the relationship between final-assembly/envelopment and axonal transport of virus particles [16]: do non-enveloped particles in axons represent progeny particles that are not yet enveloped, or inoculum particles that have already shed their envelopes during entry?

Campenot trichambers, like related microfluidics devices [17-19], are an

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important tool to clarify this problem of distinguishing virus inoculum and viral progeny (*see* **Note 1**). These compartmented neuron culture devices allow SCG and DRG neurons to extend axons between chambers, while maintaining fluidic separation between the chambers [20]. The "trichamber" version separates the culture volume into three compartments. Dissociated neurons can be seeded in one chamber, and over the course of approximately two weeks *in vitro*, axons can extend underneath two chamber walls into the opposite chamber [21, 22]. Using a lipophilic dye to measure axonal penetrance, anywhere from ~15-50% of neurons extend axons under both chamber walls [13, 23] (*see* **Note 2**). At this point, a virus infection experiment can be performed. For example, virus inoculum can be added to the cell body compartment to initiate infection, and following virus replication, progeny particles can be studied in the fluidically-isolated axons in the opposite chamber. Because the chambers are fluidically-separated, only progeny particles can reach the axon chamber via anterograde axonal transport processes. This ability to observe viral progeny without interference from the inoculum is important to better understand the intracellular transport, egress, and spread of neuroinvasive viruses.

2 Materials

2.1 Full List of Materials

1. 35-mm plastic tissue culture dishes
2. Ibidi µ-dish, 35 mm, high (81156, Ibidi) (see section 3.2.1)
3. Boric acid
4. Sodium tetraborate

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5. Poly-DL-ornithine hydrobromide (see section 2.2, step 2)
6. 0.2µm filters, vacuum-driven and/or syringe-driven
7. HBSS (-Ca, -Mg, +phenol red) (*see* **Note 3**)
8. Laminin, natural mouse (23017015, Invitrogen) (see section 2.2, step 3)
9. Neurobasal medium (21103049, Invitrogen) (see section 2.2, steps 4 and 6)
10. B27 supplement (50X), serum free (1750444, Invitrogen)
11. Penicillin-streptomycin-glutamine (100X)
12. NGF mouse 2.5S native protein
13. Water, tissue culture grade or ultrapure deionized
14. DMEM, powder
15. Methocel E4M (Hydroxypropylmethylcellulose, Hypromellose 2910 USP, viscosity 4000 cP at 2% in H_2O, H7509 Millipore-Sigma) (see section 2.2, steps 5-6)
16. Pin rake (Tyler Research)
17. Campenot trichambers (CAMP320, Tyler Research)
18. Reagents and equipment for cleaning Campenot chambers (see section 3.4)
19. Hemostat forceps, 90° curved tip
20. Silicone high-vacuum grease, Dow Corning (59344−055, VWR) (see section 2.2, step 1)
21. Syringe, 10mL disposable
22. Syringe, 3mL disposable Luer-lock (see section 2.2, step 1)
23. Sawed-off 18G hypodermic needle (*see* **Note 4**) or blunt-fill needle (305181, BD) (see section 3.2.2)
24. Rat, Sprague-Dawley, E15-E18 timed pregnant

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25. Forceps, fine tip Dumont #5
26. Knives, microdissection
27. Hibernate-E Medium (A1247601, ThermoFisher)
28. Trypsin (no EDTA, specific activity ≥10,000 BAEE units/mg) (see section 2.2, step 7)
29. Pasteur pipets, 14.6cm (see section 2.2, step 8)
30. AraC (cytosine β-D-arabinofuranoside hydrochloride) (see section 2.2, step 9)

2.2 Materials prepared in advance

1. **Silicone vacuum grease loaded into 3mL Luer-lock syringes:** Initially load the silicone vacuum grease into a 10mL syringe. Remove the plunger from the 10mL syringe, align the opening of the silicone vacuum grease tube to the back of the 10mL syringe, fill the syringe, and replace the plunger. Next, use the 10 mL syringe to fill the 3mL syringe. Remove the 3 mL syringe plunger and fill the barrel with grease. Replace the plunger of the 3mL syringe and squeeze out any excess air and grease so that the plunger is correctly seated. Wrap the grease-filled 3mL syringe in aluminum foil and autoclave on a liquid cycle. While only one syringe is sufficient to assemble trichambers, it is recommended to prepare multiple grease-filled 3mL syringes in advance and store them at room temperature until use.
2. **Poly-ornithine solution (500µg/ml in borate buffer)**: Make a 0.1M sodium borate solution: Dissolve 19.1g sodium tetraborate to 500mL ddH_2O. Make a 0.1M boric acid solution: Dissolve 6.2g boric acid in 1L ddH_2O. Make boric acid buffer: Beginning with 460mL of boric acid solution, titrate in approximately 40mL of sodium borate solution to pH 8.3. Make poly-ornithine solution: Dissolve 100mg poly-DL-ornithine hydrobromide

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into 200mL of borate buffer. Filter sterilize, aliquot into 50mL conical tubes, store frozen at -20°C, and thaw prior to use. Leftover poly-ornithine solution can be re-frozen and used for up to two freeze-thaw cycles.

3. **Laminin solution (10µg/mL in HBSS):** Thaw an aliquot of laminin and dilute 1:100 in HBSS, to a final concentration of 10ug/mL. Make fresh each time and make the minimal amount required to coat dishes.
4. **Complete neuronal medium**: It is recommended to make complete neuronal medium in 50mL batches. In a 50mL conical tube, mix 48mL of Neurobasal medium, 1mL of 50X B-27 supplement, 0.5mL of 100X glutamine/penicillin/streptomycin solution. Filter sterilize using a 0.2µm syringe-driven filter fitted to a 60mL syringe. Add 30-50µL of NGF after filter sterilizing (60-100 ng/mL final concentration). Use immediately, or store up to two weeks at 4°C.
5. **1% or 2% methocel in DMEM**: DMEM thickened with methocel is required in two different concentrations: 1% methocel in DMEM is used during the during the process of assembling Campenot trichambers (see section 3.2. step 6); 2% methocel in DMEM is used to make methocel-thickened neuronal medium (see step 6 below). Make a 2% or 4% (w/v) solution of methocel in water: In a 500mL autoclavable bottle, add a magnetic stir bar, 2g or 4g methocel, and 100mL of tissue culture grade or ultrapure ddH_2O. Autoclave on liquid setting. Methocel is insoluble at high temperatures and is very slow to rehydrate and dissolve after autoclaving. Stir at room temperature or 4°C until dissolved, which may take several days. Make a 7.5% (w/v) solution of sodium bicarbonate: Dissolve 7.5g sodium bicarbonate in 100mL of tissue culture grade or ultrapure ddH_2O. Filter sterilize. Make a 2X DMEM solution: Follow manufacturer's

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instructions to measure the appropriate amount of DMEM powder to make 1L of medium 1X DMEM, but dissolve in 500mL to make a 2X DMEM solution. Autoclave or filter sterilize. Add 15mL of 7.5% sodium bicarbonate solution. Phenol red pH indicator in the solution should show a bright red color. Make methocel in DMEM: Mix 25mL of the methocel in water solution with 25mL of 2X DMEM, for a final concentration of 1% or 2% methocel in 1X DMEM (*see* **Note 5**). When making 1% methocel in DMEM for assembling Campenot chambers, adding penicillin/streptomycin is optional. Store at 4°C for months.

6. **Neuronal medium containing 1% methocel:** Prepare an appropriate volume of neuronal medium (see section 2.2, step 4, above), except with double the typical concentration of B-27 and glutamine/penicillin/streptomycin supplements. Filter sterilize. Add double the typical concentration of NGF, 200ng/mL. Combine this Neurobasal medium with 2X supplements with an equal volume of 2% methocel in DMEM (see section 2.2, step 5, above). Mix by pipette or inverting tube several times. The resulting medium is a 1:1 mixture of Neurobasal and DMEM medium, containing 1% methocel, and the typical concentration of supplements as in complete neuronal medium (*see* **Note 5**). Store up to 10 days at 4°C.
7. **2X Trypsin solution:** Dissolve lyophilized trypsin in HBSS at a concentration of 0.5mg/mL. Filter sterilize. Divide into 500μL single-use aliquots and store at -20°C (*see* **Note 6**).
8. **Flame-polished Pasteur pipets:** Partially close the air intake of a Bunsen burner for a cooler yellow/orange flame, which will provide more control over the flame-polishing process (optional). Pass the tip of the Pasteur pipet though the flame until the glass just

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begins to melt. As the glass melts, the opening of the pipet will become constricted. Continue to melt the tip slowly until the opening of the pipet tip is approximately half of its original diameter. Make flame-polished Pasteur pipets in batches and autoclave.

9. **AraC antimitotic solution**: Prepare a 1mM stock solution in DMEM: Dissolve 25mg of cytosine β-D-arabinofuranoside HCl into 89.4 mL DMEM (*see* **Note 7**).

3. Methods

The assembly of Campenot trichambers onto cell culture dishes requires finesse, and there are several common defects that can occur: Typically, a fraction of trichambers leak, or alternatively, trichambers can be too tightly sealed, preventing axonal penetration. Neuronal cultures can also fail over the course of 2-3 weeks required for robust axonal penetration. With these difficulties in mind, it is important to assemble more trichambers than required for planned experiments.

See **Note 8** for a summary schedule of the following procedures.

3.1 Preparation of 35mm Cell Culture Dishes

1. Two days before trichamber assembly, begin coating the culture dishes. Add enough poly-ornithine solution to cover the cell culture area of each 35mm dish, and incubate overnight in a cell culture incubator (37˚C, 5% CO_2, humidified).
2. The next day, remove the poly-ornithine solution, rinse once with tissue grade/ultrapure water or HBSS, and replace with enough laminin solution to cover the cell culture area, and incubate overnight in a cell culture

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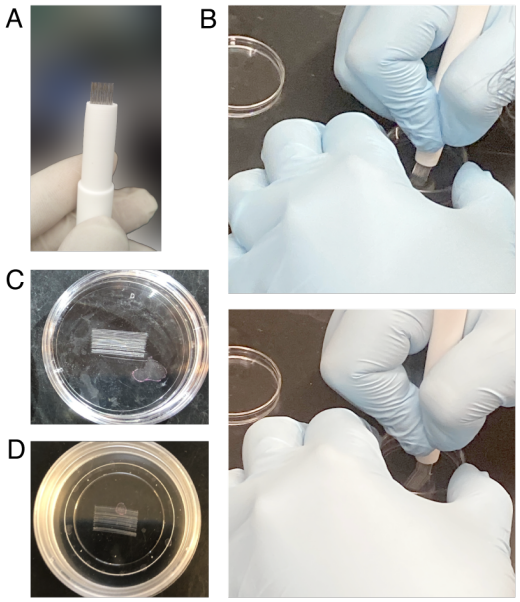

Fig. 1 Preparing cell culture dishes. (**a**) Commercially-available pin rake (Tyler Research). (**b**) Using the pin rake to scratch an array of parallel grooves on the dish. Hold the pin rake at an angle and firmly press the pins into the plastic surface so the grooves are etched deeply. (**c**) Example of resulting grooves on a standard 35mm culture dish. (**d**) Example of resulting grooves on an Ibidi µ-Dish. (**c**-**d**) Note the length and position of the grooves in the center of the culture area, particularly their position within the optical plastic inset of the Ibidi µ-Dish. Also, note the presence of a splotch of dried HBSS on each dish, indicating that the dish is fully dried.

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incubator. If necessary, the laminin incubation can be reduced to 6 hours.

3. The next day, gently remove the laminin solution and gently rinse dishes 3 times with HBSS. After the last rinse, leave the dishes open in the biological safety cabinet ~10 min until dry. Look for a splotch of dried salt residue on the plastic surface (visible in Figure 1C-D) to be sure dishes are completely dry.

3.2 Assembling Campenot trichambers

1. Sanitize the pin rake with 70% ethanol and dry, then scratch across the cell culture area in the middle of the dish, forming an array of parallel grooves, approximately 1.2cm long (Figure 1). The grooves must be long enough to pass under both inner chamber walls. Be firm! If the scratches are too shallow, the vacuum grease will form too tight of a seal, and axon penetration will be blocked or reduced. Figure 1C-D shows correctly scratched dishes.
2. Sanitize a hemostat or blunt forceps with 70% ethanol, and attach the blunt 18G needle to the autoclaved 3mL grease-filled syringe, and depress plunger until a bead of grease is expelled.
3. Clamp the hemostat onto one of the inner trichamber walls, and lock the hemostat on the first notch (do not lock the hemostat any tighter because this can damage the trichamber wall). Lay the hemostat on the benchtop so the trichamber ring is facing up (Figure 2A). Using a sterile p1000 pipette tip, push the trichamber so that it is level, parallel to the benchtop.

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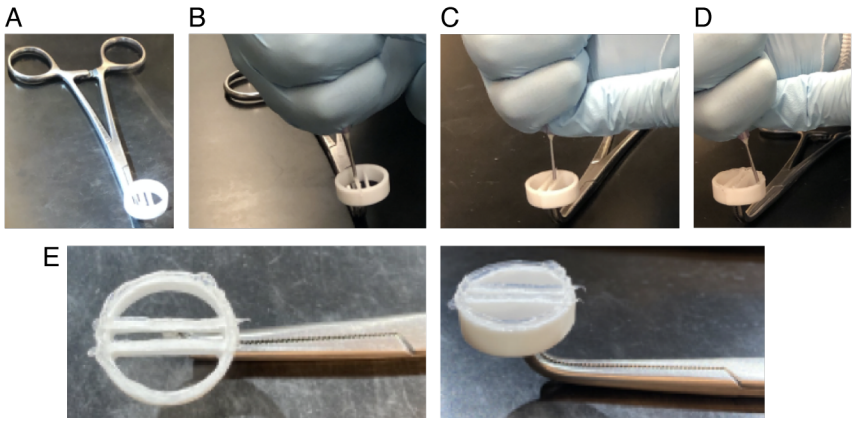

Fig. 2 Greasing the Campenot trichamber ring. (**a**) A trichamber ring held level in a hemostat. (**b-c**) Applying a bead of silicone grease to the two inner chamber walls. Note the sawed-off or blunt needle used to dispense the grease. (**d**) After applying grease to the inner chamber walls, apply grease to the outer chamber ring. (**e**) Example of resulting greased trichamber.

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Leveling the trichamber is important for subsequent greasing and assembly steps.

4. Using the grease-filled syringe, apply a bead of grease along the trichamber inner walls (Figure 2B-C). Hold the syringe at a 45° angle to the trichamber walls, and dispense the grease slowly and evenly. Do not use pressure to stick the grease to the chamber walls; rather, dispense a bead of grease and allow it to lay onto the chamber wall under its own weight. It is important to dispense the grease evenly, so that there is a uniform unbroken bead of grease along the inner chamber walls. If the bead of grease has imperfections, it can be gently scraped off with the blunt needle tip or a sterile pipette tip to try again. The quality of the grease barrier on the inner chamber walls is critical to avoid leaks while still allowing axonal penetration.
5. Next, apply a bead of grease around the outer circular chamber wall (Figure 2D). More grease can be used on the outer wall, as this does not affect axonal penetration. Overlap the ends of the bead of grease to make a closed loop with no gaps. If necessary, apply two beads of grease to ensure the outer wall does not leak. Figure 2E shows a correctly greased trichamber ring.
6. Retrieve a prepared culture dish, and pipette a long drop (approximately 200μL) of 1% methocel DMEM onto the array of grooves (Figure 3A). The drop of methocel medium must be big enough to span the two inner chamber walls. This layer of methocel medium is critical to allow the axons

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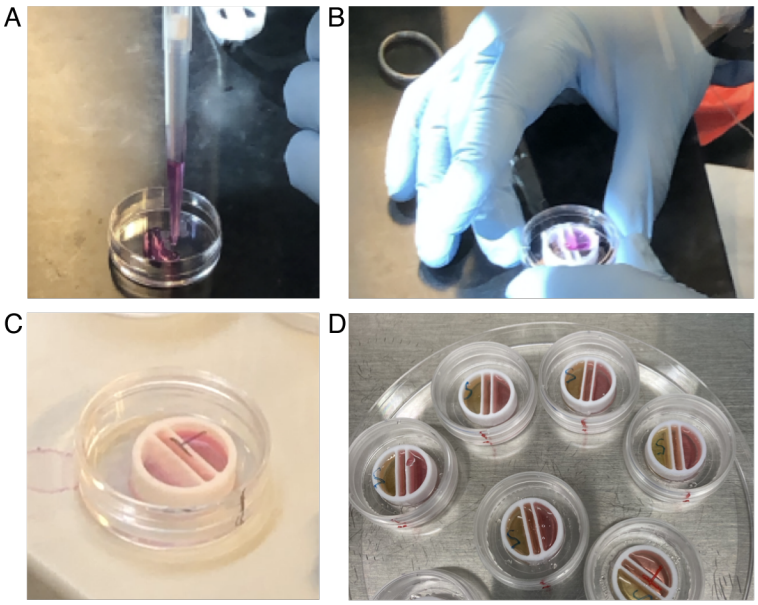

Fig. 3 Mounting the greased trichamber ring in the prepared cell culture dish. (**a**) Pipetting methocel-thickened DMEM to cover the etched grooves on the culture dish. (**b**) Placing the culture dish gently onto the greased trichamber held in the hemostat. (**c**) Example of assembled Campenot trichamber with neuronal medium added to the middle compartment to check for leaks. Note the mark on the side of the dish, which indicates orientation, and the T mark on the lid, which indicates a leak under one of the inner chamber walls. (**d**) Example of multiple Campenot trichamber cultures. Note the S mark of the lids, which indicates the cell body (soma) compartment, and the yellower color of the medium in the cell body compartment, which indicates no leaks have developed.

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to penetrate under the chamber walls. Proceed with the next steps immediately so the methocel does not dry.

7. Flip the dish over and place it gently on top of the trichamber (Figure 3B), with the grooves arranged perpendicular to the inner walls of the trichamber. Do not apply any pressure; just let the dish lay onto the trichamber under its own weight. Lightly tap the hemostat 3-4 times on the work surface to attach the greased trichamber to the dish. Flip the hemostat over, unlock it, and allow the trichamber and culture dish to fall approximately 1-2cm onto the benchtop. This tapping and dropping procedure applies enough force to allow the grease on the outer wall to seal against bare plastic, but does not cause the inner walls to be too tightly sealed.
8. Closely inspect the dish to ensure the grease between the dish and outer trichamber walls is completely attached with no gaps. If the outer walls are incompletely sealed to the dish, drop the dish 1-2cm a few more times or apply very light pressure using a sterile pipette tip.
9. To check for leaks and prevent the methocel medium from drying, add complete neuronal medium (~250 µL) to the middle compartment, cover, and set aside while assembling the remaining trichambers. If the medium leaks under both inner chamber walls, the assembled trichamber cannot be used. If the medium leaks under only one of the two inner walls, the trichamber might still be used, depending on experimental demands. It is helpful to mark the dishes to keep track of which of the two inner barriers

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is compromised.

10. Once assembled and checked for leaks, fill all three compartments with complete neuronal medium. Dissociated neuronal can be plated immediately, if available, or the trichambers can be stored overnight, at room-temperature or in a cell culture incubator. Figure 3C shows a correctly assembled Campenot trichamber containing neuronal medium.

3.2.1 Variation: Optical Plastic Campenot Trichambers

To facilitate microscopy using high-magnification oil-immersion objectives, it is possible to assemble Campenot trichambers in tissue culture dishes with optical plastic coverslips (Ibidi µ-Dish). It is important to use the Ibidi µ-Dish with high side walls, to accommodate the height of the Campenot chambers. Assembly of optical plastic trichambers is the same as for standard 35mm dishes, with two major exceptions: First, when scratching parallel grooves with the pin rake, the optical plastic coverslip will flex under the pressure of the pin rake, so greater force is required to scratch the plastic surface deeply enough. Second, when mounting the greased trichamber onto the Ibidi µ-Dish, the trichamber must be carefully centered within the recessed area containing the optical plastic coverslip. Placement outside the recessed optical plastic area will compromise the grease barriers and cause leaks. Figure 1D shows a correctly scratched Ibidi µ-Dish.

3.2.2 Variation: 3D Printed Campenot Trichambers on Glass-Bottom Dishes

Another option is to use a 3D printer to fabricate the trichamber onto the tissue

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culture dish [24]. This method used a custom-designed 3D printer capable of extruding a wide variety of materials, including polymers to form grooves and the trichamber walls, silicone vacuum grease, and even cells suspended in culture medium. Importantly, this 3D printing method allowed the authors to fabricate trichambers on cell culture dishes with glass coverslips (e.g. MatTek dishes), which cannot be scratched with the pin rake (as in section 3.2, step 1, above).

Assembling trichambers by hand requires experience, it is laborious and time-consuming, and trichambers vary in axonal penetration. 3D printing mitigates each of these limitation by automating the process. However, this method currently requires specialized 3D printing expertise, custom 3D printer devices that are capable of extruding a variety of materials, and is limited to using particular materials that are biocompatible and can be sterilized for cell culture applications, limiting its wider use.

3.3 Primary Superior Cervical Ganglia (SCG) and Dorsal Root Ganglia (DRG)

1. Euthanize a E15-18 timed-pregnant rat in accordance with all applicable animal care and use regulations. Euthanasia via CO_2 inhalation is preferred to maintain viability of peripheral ganglia.
2. Wet the abdominal fur with 70% ethanol.
3. Using sterile surgical scissors, open the abdomen and remove the uterus.
4. Using surgical knives and forceps, open the uterus and remove individual embryos. A detailed protocol for dissecting SCGs can be found in Ch'ng et al. 2005 [25] and a detailed protocol for dissecting DRGs can be found in Fenstermacher, et al. [18].

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5. For convenience and flexibility, dissected ganglia can be stored in Hibernate-E medium at 4°C for 1-2 weeks; however, neuron viability gradually decreases during storage. As an added benefit, storing ganglia in this way also strongly reduces the viability of non-neuronal cells that would otherwise proliferate until antimitotic AraC treatment (see section 3.5, step 1).

3.4 Dissociation of Peripheral Ganglia

1. Protein-coat the inside of a flame-polished Pasteur pipet, otherwise ganglia and dissociated neurons will stick to the glass. Fill the pipet with a protein-rich solution, and set aside during subsequent steps. This protein-rich solution can be 2-10% bovine serum albumin (BSA) in a saline buffer, fetal bovine or calf serum (FBS/FCS), cell culture medium containing 10% serum, or the HBSS containing blood and tissue debris from dissecting the ganglia.
2. At this point, ganglia will be in either HBSS containing some blood and tissue debris from dissection, or stored in Hibernate-E medium (see section 3.3, step 5). In either case, wash the ganglia by adding HBSS to fill, centrifuge at 1200 x g for 1 min to pellet the ganglia, and then remove most of the HBSS, leaving approximately 0.5mL. Gently flick and swirl the tube to dislodge the ganglia from the bottom of the tube.
3. Add 500µL of trypsin, swirl to mix, and incubate 15 min at 37˚C.
4. Wash by adding HBSS to fill, centrifuge at 1200 x g for 1 min to

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pellet, and gently remove HBSS, being careful to not aspirate the ganglia. Repeat a total of three times to remove residual trypsin.

5. After removing the final HBSS wash, add 100µL of complete neuronal medium per ganglion, but not less than 500µL.
6. Using the protein-coated flame-polished Pasteur pipet, vigorously pipet up and down so that the ganglia break up into a uniform cell suspension. Try to minimize bubbling/foaming while pipetting. Triturating the ganglia too much will reduce neuron viability. Compared to SCGs, thicker DRG roots (the pre- and post-ganglionic nerves) do not break apart easily, so DRG suspensions are typically less homogenous.
7. Plate the resulting cell suspension into one compartment of the assembled Campenot trichambers. For rat SCGs, two-thirds of a ganglion per trichamber is sufficient. For mouse SCGs, use 1-1.5 ganglia per trichamber. For rat DRGs, use 1-2 ganglia per trichamber.

3.5 Maintaining Campenot trichamber cultures

1. SCG and DRG cultures must be treated with an antimitotic drug to suppress the proliferation of non-neuronal cell types. Add 1µL of AraC solution to the cell body compartment. AraC can be added immediately to DRG cultures; however, embryonic SCG neurons sometimes undergo one round of cell division in vitro, so it is recommended to add AraC to SCG cultures after two days in vitro.

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Depending on the density of non-neuronal cells, cultures may need to be treated with AraC during subsequent media changes as well.

2. Change medium every 3-5 days. Using a P1000 pipette tip, gently remove about two-thirds of the media volume from all three compartments. To avoid disturbing the neurons, be sure to leave the culture area completely covered with medium. Gently fill the compartments with fresh pre-warmed complete neuronal medium. Allow the medium to drip gently down the chamber walls, but do not touch the trichamber walls with the pipette tip.
3. Before each medium change, observe the color of the phenol red pH indicator in the neuronal medium. The medium in the cell body compartment will acidify due to cell metabolism, resulting in a yellower color, whereas the compartments that do not contain cell bodies will remain a redder color (Figure 3D). This color difference between compartments is a helpful indication that no leaks have developed.
4. Axons will begin to extend from neuronal cell bodies within 24 hours, and will penetrate under both inner walls by 2 weeks in vitro. Axonal extension can be monitored by standard phase contrast cell culture microscope (Figure 4).
5. Experiments can be performed between approximately 2-4 weeks in vitro. Prior to performing a virus infection experiment, be sure the medium has been changed several times without adding AraC,

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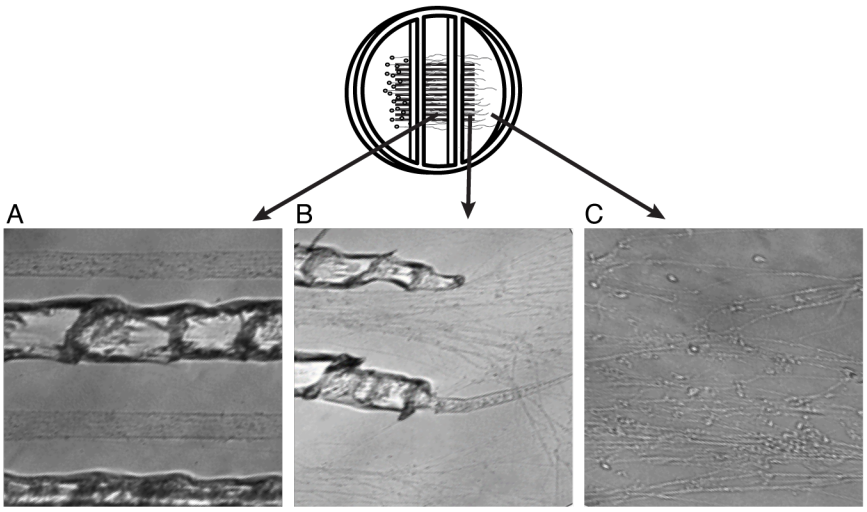

Fig. 4 Axons that have penetrated the inner chamber walls are visible by phase contrast microscopy. Axons of DRG neurons shown here, but axons of SCG neurons appear essentially the same. (**a**) Axons in the middle chamber tend to form a tight bundle (fascicle) between the grooves. (**b-c**) Where the grooves end in the axon compartment, axons fan out over the culture surface, no longer forming a tight bundle. Experiments are typically performed at this stage of axonal outgrowth, after about 2-3 weeks in culture.

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because residual AraC can inhibit viral DNA replication. The medium in the middle compartment can be replaced with neuronal medium containing 1% methocel to further reduce the risk of leaks (optional).

3.6 Cleaning the trichambers

1. Campenot trichamber rings are made from Teflon, which can be cleaned and reused for years. Observe all applicable biosafety procedures, particularly if the trichambers have been used with hazardous viruses or neurotoxins.
2. First, remove trichamber rings from cell culture dishes using blunt forceps or hemostat.
3. Drop the trichamber rings into a bottle containing a 70% ethanol. If biohazardous agents are not effectively inactivated by ethanol, a 10% bleach solution can be used instead. Ensure sufficient contact time to inactivate biohazardous agents.
4. Drain the ethanol solution and manually wipe trichamber rings with low-lint paper towels/tissues to remove excess silicone grease.
5. Place the trichamber rings in a clean bottle with deionized water and bring to a boil in a microwave. Carefully shake the bottle. Depending on how much grease residue remains on the trichamber rings, grease droplets may make the water cloudy. Replace the deionized water and repeat, if necessary, until the water remains clear.

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6. Drain the water and spread the trichamber rings on a low-lint paper towel to dry.
7. Autoclave the clean trichamber rings prior to re-use.

4. Notes

1. In classic studies investigating the infection and spread of neurotropic alpha herpesviruses, such as HSV-1 and pseudorabies virus, Campenot trichamber cultures were used to separate virus inoculum that initiated infection in one compartment, from progeny virus released in another compartment. To study post-entry retrograde axonal transport and subsequent establishment of infection, virus inoculum can be added to the axon compartment, and after a full replication cycle, progeny virus can be titered from the cell body compartment. Alternatively, to study post-replication axonal sorting and transport, as described in the introduction, viral infection can be initiated in the cell body compartment and progeny virus titered in the axonal compartment. Typically, a monolayer of non-neuronal cells is added to the axonal compartment to amplify virus to more easily detectable amounts. Detailed procedures for these types of experiments, infecting and measuring virus spread through neurons via titering, are available from Curanović, et al. [22].

In addition, Campenot trichamber cultures have allowed the compartmentalization not only of viral inoculum, but also compartmentalization of various experimental manipulations, such as drugs or signaling molecules. For example, cytokines and drugs can have dramatically different effects on entry, axonal transport, and establishment of infection depending on whether they are applied to the cell bodies or axons. These types of experiments have been important to better mimic

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the spatial and fluidic separation between axons and cell bodies that occurs in vivo, leading to a better understanding of infection and establishment of viral latency in neurons [13, 26-28].

2. Retrograde labeling of neurons using fluorescent lipophilic dyes is a powerful method to quantify axonal penetration and identify particular neuron cell bodies that have extended their axons through the trichamber [13, 22, 37]. To illustrate the key factors that govern how axons extend through the Campenot trichambers, we performed two-color fluorescent lipophilic dye experiments, comparing the standard Campenot trichamber (Figure 5A) to an alternative chamber format (Figure 5B-D). In the standard Campenot trichamber format, we added the fluorescent lipophilic dyes DiO or DiI (V22885 or V22886, Invitrogen) to the middle or right axonal chambers, respectively. Neuronal cell bodies were then imaged by phase contrast and fluorescence microscopy to identify DiO-labeled (green) and DiI-labeled (red) cells. Neurons that extended axons under both inner chamber walls exhibited a combination of red and green fluorescence; however, many neurons showed only green fluorescence, indicating that their axons had penetrated only as far as the middle chamber (Figure 5A). This result shows that the chamber walls are a significant barrier to axonal penetration.

In the alternative trichamber design, instead of a single array of grooves that span both inner chamber walls, we produced two separate, shorter sets of grooves, each spanning only one of the inner chamber walls (Figure 5). We then plated dissociated SCG neurons in the middle compartment, so that these cells can extend axons into either the right or left chamber by extending under only one chamber wall. DiO or DiI were added to the left or right axonal chambers, respectively. Over four

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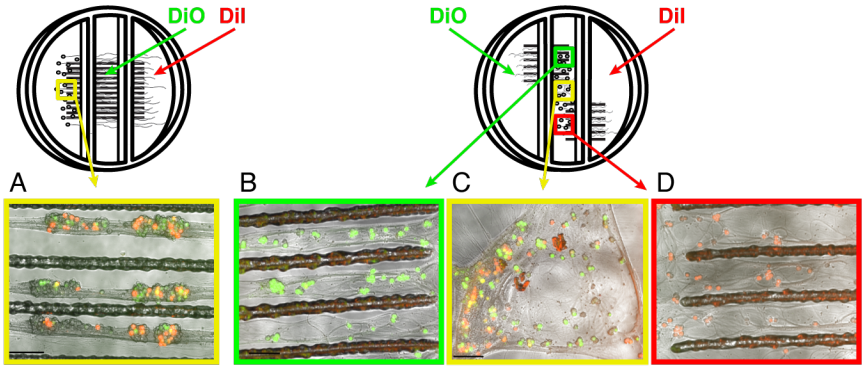

Fig. 5 Investigating axonal penetration (*see* **Note 2**). Schematics represent either the standard or an alternative trichamber format. Alternative trichambers have two separate sets of grooves, extending under the left or right inner chamber walls, with SCG neurons plated in the middle compartment. Axonal penetration is assessed using fluorescent lipophilic dyes, DiO (green) and DiI (red). (**a**) Merged fluorescent and phase contrast images of cell bodies in the standard trichamber, labeled by DiO in the middle compartment and DiI in the right axonal compartment. (**b-d**) Merged fluorescent and phase contrast images of cell bodies in the middle compartment of the alternative trichamber. Image areas are indicated in the trichamber schematics above.

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independent replicates, 76.4 ± 14.1% (mean ± s.d.) of neurons were dye-labeled, indicating that their axons penetrated one chamber wall into either the left or right chamber. By comparison, typically 15-25% [22, 37], up to a maximum of 50% [13], of neurons extend axons under both chamber walls in the conventional trichamber format. Importantly, whether the cell bodies were DiO- or DiI-labeled depended strongly on the proximity to the grooves extending under the middle chamber walls. Cell bodies between the grooves extending to the left were almost exclusively DiO-labeled (green, Figure 5B), whereas cell bodies between the grooves extending to the right were almost exclusively DiI-labeled (red, Figure 5D). Neurons in the middle, between the two sets of grooves, were roughly divided with some DiO-labeled and some DiI-labeled cell bodies (Figure 5C). These data show that the pin rake-scratched grooves are critical for guiding axons towards the chamber walls and allowing them to penetrate under the chamber walls.

3. It is important to use a HBSS formulation that does not contain calcium or magnesium, because these ions will inhibit enzymatic dissociation of the ganglia and may cause excitotoxicity in neurons.

4. We use sawed-off 18 gauge needles to dispense silicone vacuum grease. To cut the needle, we use a Dremel rotary tool fitted with a metal cutting/grinding disc. The needles cannot be cut with scissors, metal shears, or wire-cutters, because these tools will crimp the needle. It is important that the cut opening is smooth, without any remaining metal burrs. We store the cut/blunt needles in 70% ethanol when not in use.

5. If it is more convenient, 2% methocel in DMEM can be diluted with standard (1X) liquid DMEM to make 1% methocel in DMEM for chamber assembly. To make neuronal

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medium with 1% methocel, it is also possible to make 4% methocel in DMEM and then dilute it 1:3 with neurobasal medium. When diluting any methocel-thickened medium, be sure to mix well to ensure a uniform viscosity.

6. It is possible to make the trypsin solution at other stock concentrations, such as 10X (2.5mg/mL) or 1X (0.25mg/mL), as long as the final working concentration on the ganglia is 0.25mg/mL. Trypsin from other manufacturers may have a different specific activity, so concentrations may need to be adjusted. Do not use common cell dissociation solutions that contain EDTA.

7. Cytosine β-D-arabinofuranoside in the form of HCl salt is preferred because it is available in smaller 25mg quantities from Millipore-Sigma. It is possible to use cytosine β-D-arabinofuranoside that is not an HCl salt, but you will need to adjust molarity calculations to account for the slightly different molecular weight.

8. Summary of procedures with suggested schedule. Ensure that materials and reagents listed in section 2.2 are prepared in advance. Clean and autoclave Campenot trichamber rings, dissection tools, etc.

Day 1: Sacrifice pregnant rat and dissect embryos. Store peripheral ganglia in Hibernate-E at 4°C. Based on number of ganglia available, plan experiments to determine how many trichamber dishes to assemble (plan to assemble more than needed). Add poly-ornithine solution to 35mm dishes and incubate overnight.

Day 2: Replace poly-ornithine solution with laminin solution, and incubate overnight. If necessary, laminin coating time to 6 hours and proceed immediately with next steps.

Day 3: Rinse dishes, air-dry, and scratch with pin-rake. Assemble trichambers on

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dishes by greasing a trichamber, apply a drop of 1% methocel DMEM, mount the trichamber, add complete neuronal medium to middle compartment to check for leaks. Once all trichambers are assembled and filled with complete neuronal medium, store overnight or proceed immediately with next steps.

Day 4: Dissociate ganglia and plate in the assembled trichambers. If using DRG neurons, AraC can be added to the culture medium immediately.

Day 6: Observe cultures by phase contrast microscopy to assess neuronal viability, axonal outgrowth, and proliferation of non-neuronal cells. Two days after plating SCG neurons, add AraC to the culture medium.

Day 8: Observe cultures by phase contrast microscopy and change medium. Add AraC to the culture medium again if non-neuronal cells remain. Observe cultures and change medium every 3-5 days thereafter.

Day 18-25: Observe cultures by phase contrast microscopy to assess axonal penetration through the chamber barriers. Campenot trichamber cultures are now ready for experiments.

9. While the classic approach of measuring virus spread in the Campenot trichamber by titering progeny virus output has provided many insights into virus infection and spread in neurons, this approach is cumbersome. Titering by serial dilution plaque assay takes days, and only a single timepoint post-infection can be measured per trichamber culture. For basic molecular and cell biological studies, using recombinant viruses expressing genetically-encoded fluorescent proteins allows virus infection and spread to be assessed over time by fluorescence microscopy. Detailed approaches and design considerations for making recombinant alpha herpesviruses expressing fluorescent

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proteins can be found in Hogue, et al. 2015 [10].

Figure 6 illustrates an example of this approach. HSV-1 recombinants expressing a red fluorescent protein fused to the small capsid protein (mRFP-VP26) [29] were generated by viral homologous recombination, as previously described [10, 14]. HSV-1 17syn(+) mRFP-VP26 (also known as HSV-OK14) was generated using the common laboratory strain, 17syn(+), that exhibits wild-type anterograde axonal trafficking and spread. HSV-1 MacIntyre mRFP-VP26 (also known as HSV-425) contains mutations in viral membrane proteins gI (US7) and US9 [30], which are required for anterograde axonal trafficking and spread in neurons. SCG neurons were cultured in Campenot trichambers for 2-3 weeks, until axons were observed extending into the right axonal chamber by phase contrast microscopy. One day before infection, ~10^4 Vero cells (CCL-81, ATCC) were plated into the axonal chamber. The cell body compartments were then infected with HSV-1 recombinants at a high multiplicity of infection, and virus replication and spread was monitored over 48 hours by fluorescence microscopy. Both recombinants efficiently infected neuronal cell bodies (Figure 6, left images). Consistent with their expected phenotypes, HSV-1 17syn(+) mRFP-VP26 exhibited robust anterograde axonal spread to the axonal compartment, whereas the mutant HSV-1 MacIntyre mRFP-VP26 was not able to spread to the axon compartment (Figure 6, right images). Importantly, axons are generally unable to cross the grooves etched by the pin rake (see also Figure 4 and **Note 2**); therefore, virus spread was also restricted to particular “lanes” between the etched grooves (Figure 6, top-right image). Altogether, these results illustrate the usefulness of genetically-encoded fluorescent proteins and fluorescence microscopy to assess viral spread in Campenot trichamber cultures.

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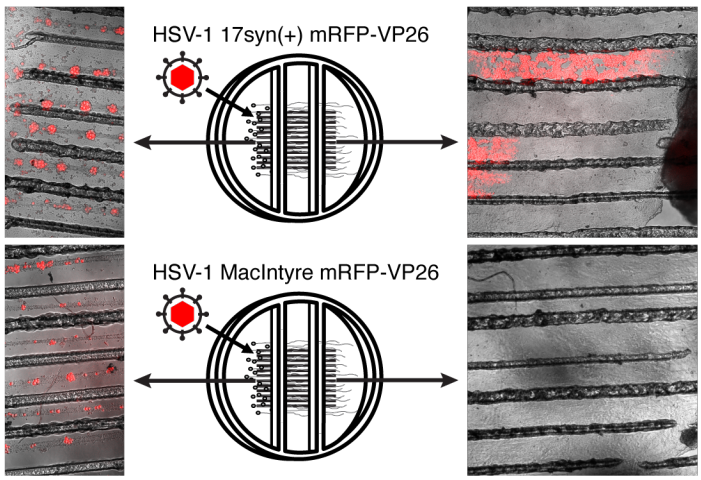

Fig. 6 Monitoring virus spread in the Campenot trichamber using genetically-encoded fluorescent proteins (*see* **Note 9**). SCG neurons were cultured in Campenot trichambers for 2-3 weeks, and a monolayer of amplifying Vero cells was plated in the axon compartment. The cell body compartment was then infected with HSV-1 recombinants expressing a red fluorescent protein fusion to the small capsid protein, VP26 (mRFP-VP26). The HSV-1 17syn(+) parental strain exhibits wild-type anterograde axonal spread in trichamber cultures (top row), whereas the mutant, HSV-1 MacIntyre, is unable to traffic progeny particles via the axons, and cannot spread though the trichamber (bottom row).

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10. The ability to construct Campenot trichambers on optical plastic (see section 3.2.1) or glass (see section 3.2.2) allows for high-magnification live-cell fluorescence microscopy to track individual fluorescently-labeled virus particles in axons. These types of experiments allow the visualization and quantification of viral transport and individual cell-cell transmission events. For example, carefully quantifying the anterograde, retrograde, and bidirectional motility of virus particles can shed light on the ensembles of microtubule motors that mediate axonal transport of virus particles, and multicolor fluorescence microscopy allows the visualization of co-transport between virus particles and fluorescently-labeled cellular factors (e.g. [31-33]). Finally, it is possible to directly visualize cell-cell spread events, from axons to non-neuronal cells, mimicking the transmission of alpha herpesviruses from the peripheral nervous system following reactivation from latency [11]. Without the separation of inoculum and progeny virions provided by compartmentalized neuronal cultures, it would be very difficult to interpret the results of these types of experiments [16], because inoculum virus particles continue to enter and traffic in the retrograde direction in axons, even at very late times post-infection [15]. Unfortunately, even with the benefits provided by Campenot trichamber cultures, determining the direction of virus particle transport can be difficult because axons can take very circuitous paths while extending through the trichambers. For example, while the axons in the Campenot trichamber are mostly oriented with their termini towards the axon compartment, axons can sometimes make a U-turn and run in an antiparallel direction, complicating the interpretation of anterograde vs. retrograde movements.

Tracking individual virus particles in optical Campenot trichambers requires

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relatively high-end fluorescence microscopy equipment. Because axonal transport is very sensitive to temperature, an objective warmer and heated stage-top incubator or microscope enclosure are essential to maintain the cultures at 36-37°C during imaging. For longer-term imaging (>1 hr.), the stage-top incubator should also have a humidified 5% CO_2 atmosphere, or use a CO_2-independent pH-buffered cell culture medium.

Because virus particles labeled with fluorescent protein fusions are relatively dim and particles can transport in axons at velocities exceeding 1 or 2μm/s, optical components must be optimized for fast and high-sensitivity image acquisition. Ideally, the fluorescence microscope should be equipped with a 60-100X magnification, high-numerical aperture, oil-immersion objective and a high-sensitivity camera (≥95% quantum efficiency preferred, e.g. Oxford Instruments Andor EMCCD or Teledyne Photometrics Prime sCMOS cameras).

For multi-color particle tracking, switching fluorescence filters is often a severely rate-limiting process. For example, on a typical inverted fluorescence microscope (as previously described [34]) equipped with an arc lamp and motorized filter wheels, it took about 625ms to acquire sequential green and red fluorescence channels with an exposure time of 50ms each (i.e. 525ms of filter-switching overhead), limiting the acquisition framerate to 1.6 dual-color frames/s [35]. To illustrate the impact of this filter-switching lag time, we infected SCG neurons in Campenot trichambers with a pseudorabies virus recombinant (PRV-137 [36]) expressing red (mRFP-VP26) and green (gM-EGFP) fluorescent structural proteins, and imaged two-color virus particles transporting in axons (Figure 7). The lag time between fluorescence channels results in the particle moving noticeably during acquisition, leading to offset red and green puncta

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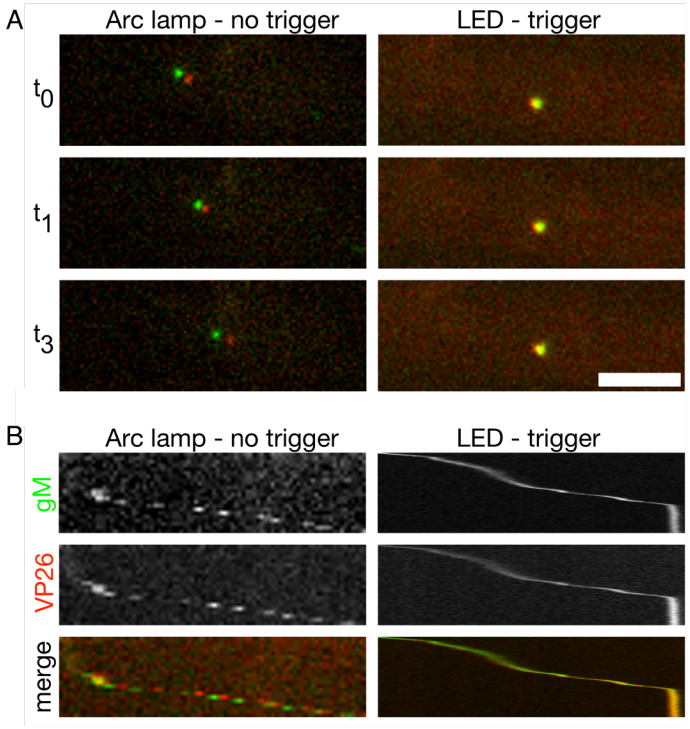

Fig. 7 Two-color fluorescence microscopy tracking individual virus particles in axons (*see* **Note 10**). SCG neurons were cultured in Campenot trichambers and infected in the cell body compartment with pseudorabies virus expressing gM-EGFP (gM, green) and mRFP-VP26 (VP26, red). 10 hours post infection, viral particles trafficking in axons were imaged in the axon compartments using mechanical filter wheel (Arc lamp–no trigger) or triggered LED illumination (LED–trigger). (**a**) Three consecutive still images taken from movies of representative virus particles transporting at ~1.5 μm/s for ~14s. Scale bar indicates 5 μm. (**b**) The same microscopy datasets displayed as kymographs (projection of X-axis movement over time). This figure was originally published by Bosse, et al. [35], and is reproduced here under the terms of the Creative Commons Attribution License.

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in still images (Figure 7A) and in kymographs projected over time (Figure 7B). To overcome this limitation, we now use a single quad-band dichroic mirror and emission filter with a multi-band laser or LED light source (e.g. Lumencor Spectra X or [35]) capable of fast triggered switching of excitation light. In our test case, using triggered LED illumination on the same microscope took 106 ms to acquire sequential fluorescence channels with the same 50ms exposure time (i.e. 6ms of overhead), allowing an acquisition framerate of 9.5 dual-color frames/s [35]. This framerate is fast enough that there is very little noticeable particle movement between fluorescence channels, so co-localization and co-transport of the green and red fluorescent markers is much clearer (Figure 7).

11. Compared to most microfluidics devices, the relatively large and open culture area of Campenot chambers facilitates their use in preparing samples for electron microscopy. For standard transmission electron microscopy, neurons can be fixed with glutaraldehyde, the trichamber ring can be carefully removed, and the sample can be stained, dehydrated, embedded in resin, and sectioned. Detailed procedures for preparing samples in Campenot trichambers for standard transmission electron microscopy are available from Curanović et al. [22].

Alternatively, to image axons by cryo electron microscopy (cryoEM), we have developed a method to grow axons directly on electron microscopy grids using Campenot trichamber cultures (Figure 8). In this method, SCG neurons were cultured in trichambers, as described in this chapter. Quantifoil gold NH2 finder grids with R2/2 or R1/4 amorphous carbon or SiO_2 support films were plasma-treated by glow discharge, sterilized, coated with poly-ornithine and laminin (as described in section 3.1), and

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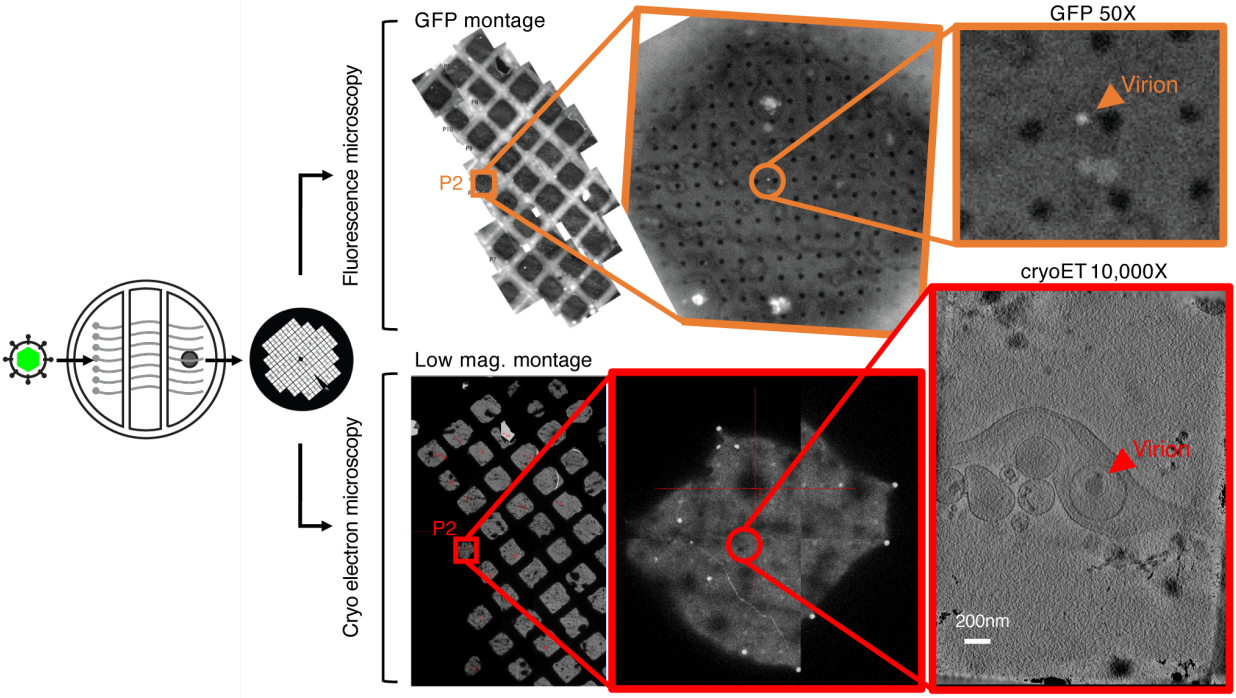

Fig. 8 Correlative fluorescence microscopy and cryo electron tomography (*see* **Note 11**). SCG neurons were cultured in Campenot trichambers, and Quantifoil gold cryoEM grids were placed in the axon compartment so that axons extend up and onto the grid. Neurons were infected in the cell body compartment with a pseudorabies virus recombinant expressing a green fluorescent protein (GFP) fusion to the small capsid protein. After plunge-freezing, grids were imaged by fluorescence microscopy to find fluorescent virus particles (top row). Regions of interest were then imaged by cryoEM and tomography to structurally characterize virus particles trafficking in axons.

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placed in the axon compartment (Figure 8). After approximately 3 weeks in culture, axons penetrated through the trichamber, and began to extend onto the support film of the Quantifoil grids, visible by phase contrast light microscopy. Neuron cell bodies were then infected with a pseudorabies virus recombinant (PRV-959) expressing a green fluorescent protein-tagged small capsid protein (mNeonGreen-VP26) [10]. At approximately 15 hours post-infection, grids were removed from the trichamber cultures, and loaded into a Leica EM GP2 plunge freezing apparatus. 1ul of a 10nm gold nanoparticle suspension was applied to the grids, grids were blotted with filter paper, and plunged into liquid nitrogen-cooled liquid ethane. Following vitrification, we performed fluorescence microscopy using a Nikon Eclipse 90i upright fluorescent microscope equipped with a 50X long working distance objective and a Linkam CMS196 cryo-stage, to locate fluorescent virus particles in axons (Figure 8, top row of images). We then performed cryo electron microscopy using a JEOL JEM2200FS electron microscope operated at 200 kV, with the objective aperture and an in-column omega energy filter set to a width of 20 eV (Figure 8, bottom row of images). After acquiring a low magnification montage, we manually aligned the fluorescence microscopy and cryoEM images based on the gold grid and holes in the support film, which were visible in both fluorescence and cryoEM images. We then collected cryoEM tomography tilt series of the regions of interest at 10,000X magnification, 6.63 Å/pixel, using a DE-20 direct electron detector recording 24 raw frames/s for a total exposure time of 0.6s per image. Tilt series were acquired semi-automatically using SerialEM software [38] in low-dose mode with a target defocus of -6µm or -8µm. For each tilt series dataset, the images were acquired from a target range of −60° to +60° in

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increments of 2°. Total cumulative dose was 60 to 70 e/Å^2. Tomographic data were reconstructed using the Etomo package in IMOD [39], using the 10nm gold beads as fiducial markers.

Most previous cryoEM tomography studies of alpha herpesviruses in axons (e.g. [40]) did not use any type of chambered or microfluidics neuron culture system; thus, it can be difficult to distinguish virus particles that are entering from those that are exiting axons. Many typical microfluidics devices have closed cell culture chambers, making them unsuitable to integrate a cryoEM grid. Thus, our example correlative fluorescence/cryoEM tomography workflow (Figure 8) illustrates the utility of Campenot trichambers for this type of experiment.

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