Video Article

Transplantation of Schwann Cells Inside PVDF-TrFE Conduits to Bridge Transected Rat Spinal Cord Stumps to Promote Axon Regeneration Across the Gap

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Abstract

Among various models for spinal cord injury in rats, the contusion model is the most often used because it is the most common type of human spinal cord injury. The complete transection model, although not as clinically relevant as the contusion model, is the most rigorous method to evaluate axon regeneration. In the contusion model, it is difficult to distinguish regenerated from sprouted or spared axons due to the presence of remaining tissue post injury. In the complete transection model, a bridging method is necessary to fill the gap and create continuity from the rostral to the caudal stumps in order to evaluate the effectiveness of the treatments. A reliable bridging surgery is essential to test outcome measures by reducing the variability due to the surgical method. The protocols described here are used to prepare Schwann cells (SCs) and conduits prior to transplantation, complete transection of the spinal cord at thoracic level 8 (T8), insert the conduit, and transplant SCs into the conduit. This approach also uses *in situ* gelling of an injectable basement membrane matrix with SC transplantation that allows improved axon growth across the rostral and caudal interfaces with the host tissue.

Video Link

The video component of this article can be found at https://www.jove.com/video/56077/

Introduction

Spinal cord injury repair is a complex and challenging problem that will require a combinatorial treatment strategy involving, for example, the use of cells and a biomaterial to provide a favorable microenvironment for transplanted cell function and axon regeneration at the site of injury. Hemisection[1,2,3,4,5,6,7,8,9] and complete transection[10,11,12,13,14,15,16,17,18,19,20,21,22] models are frequently used to assess the effects of biomaterial-based bridging therapies. The advantage of using a hemisection model is that it provides more stability for the bridging construct compared to complete transection. However, in hemisection models, it is difficult to prove axon regeneration as an outcome of the applied therapeutic method due to the presence of spared tissue. The complete transection model is the most rigorous method to demonstrate axon regeneration.

Various natural and synthetic materials have been studied for use as an injectable gel, a pre-formed gel placed in contusion or hemisection models, or as a structured conduit into hemisection or complete transection models (detailed in the reviews[23,24,25]). *In situ* gelling of an injectable matrix/SC mixture creates a more permissive interface between the transplant and the host cord for axon crossing[26,27] compared to pre-gelled matrix/SC implants[5,18,19,28]. *In situ* gelling allowed the matrix to contour around the irregular host interfaces whereas a more rigid and structured conduit or a less moldable pre-formed gel could not. A structured conduit often provides contact guidance and implant stability in contrast to an injectable matrix. The protocols presented here describe a surgical procedure that takes advantage of both an injectable basement membrane matrix (*e.g.,* matrigel, see the **Table of Materials,** referred to as injectable matrix here) and a structured conduit to evaluate axon regeneration in the most rigorous spinal cord injury model.

Electrospun poly-vinylidenedifluoride-trifluoroethylene (PVDF-TrFE) aligned fibrous hollow conduits are used in our experimental approach. PVDF-TrFE is a piezoelectric polymer that generates a transient charge when mechanically deformed and has been shown to promote neurite extension and axon regeneration both *in vitro*[29,30] and *in vivo*[31]. Electrospinning is a common scaffold fabrication method that can rapidly produce reliable fibrous scaffolds using a variety of polymers with controllable properties such as fiber alignment, fiber diameter, and thickness of the scaffold for neural and other applications[32,33,34].

Numerous studies of rat SCs transplanted into spinal cord injury sites have demonstrated treatment efficacy[5,9,18,19,20,21,26]. These transplants are neuroprotective for tissue surrounding the lesion, reduce lesion cavity size, and promote axon regeneration into the lesion/transplant site

and myelination of the regenerated axons. Human SCs can be autologously transplanted, an advantage when compared to most other neural-related cells[24]. After a peripheral nerve biopsy, SCs can be isolated and purified and will proliferate to the desired amount for transplantation into humans. Autologous SC transplantation for spinal cord injured patients has been proven to be safe in Iran[35,36,37,38], China[39,40], and the United States[41,42]. SCs are known to secrete numerous neurotrophic factors and extracellular matrix proteins important for axon growth and to play an essential role in axon regeneration after peripheral nerve injury. Our goal here is to describe methods which can investigate conduit designs to improve the outcome of SC transplantation in a complete rat spinal cord transection model.

Protocol

Female adult Fischer rats (180 - 200 g body weight) are housed according to NIH and USDA guidelines. The institutional Animal Care and Use Committee (IACUC) of the University of Miami approved all animal procedures.

1. Pre-Transplantation Preparation

1. **Conduit preparation.**
    1. Cut the conduit to 5 mm in length using a #10 blade under a dissecting microscope.
    NOTE: The inner diameter of the conduit is between 2.4-2.7 mm; the outer diameter is between 2.5-2.8 mm.
    2. Fold the conduit gently along the longitudinal side (**Figure 1A**). Cut four small incisions about 0.4 mm long and at least 1 mm from the openings of the conduit and about 1 mm apart using straight edge Vannas scissors (**Figure 1B**).
        1. Unfold the conduit (**Figure 1C**) and cut between two incisions along the same side creating two windows side by side (**Figure 1D**). Ensure that the windows can be opened and closed properly along the uncut side.
    3. Sterilize the conduits in 75% ethanol for 25 min in a 10-cm Petri dish. Rinse the conduits once for 4 min with 1x phosphate buffered saline (PBS) and transfer the conduits with sterile forceps to a new 10-cm Petri dish. Rinse the conduits twice with 1x PBS for 25 min each.
    4. Store the conduits in 1x PBS until surgery.
    NOTE: Many conduits can be sterilized at once. Store the conduits in separate sterile 1.5 mL centrifuge tubes to keep them sterile.

2. **Green fluorescent protein (GFP)-Schwann cell (SC) preparation**
    1. Prepare purified SC cultures from the tibial nerves of adult female Fischer rats as previously described[43]. At the completion of this step, plate the SCs on poly-L-lysine (PLL)-coated 10-cm Petri dishes and maintain in D10/3M medium; these SCs are defined as passage 0.
    NOTE: D10/3M medium is composed of the following: 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (pen/strep), 20 µg/mL pituitary extract, 2 µM forskolin, 2.5 nM heregulin in Dulbecco's modified Eagle's medium (DMEM).
    2. Replenish with fresh D10/3M medium (7 mL/10-cm Petri dish) every 3 to 4 days.
    3. Split SC culture once it reaches 70-80% confluence.
        1. Remove medium and rinse twice with Hank's balanced salt solution (HBSS) without calcium or magnesium.
        2. Add 5 mL of 0.25% trypsin/EDTA to each 10-cm Petri dish and incubate for 5 min at room temperature (RT).
        3. Add 5 mL of D10 medium (10% FBS and 1% pen/strep in DMEM) into a 50-mL centrifuge tube to neutralize trypsin.
        4. Gently pipet the trypsin/EDTA solution into the Petri dish several times to detach SCs. Remove the cell suspension from the dish and place it into the centrifuge tube prepared in the previous step. Rinse the dish with 5 mL of D10 medium and place it into the centrifuge tube to maximize cell harvest.
        5. Centrifuge the cells at 370 x g for 5 min at 4 ^{o}C. Remove the supernatant and resuspend the cells in 4 mL D10/3M medium for each Petri dish split.
        6. Dispense 1 mL of SC suspension and 6 mL of D10/3M medium into a 10-cm Petri dish; each Petri dish split will result in 4 Petri dishes. These SCs are defined as passage 1.
        7. Replenish with fresh D10/3M medium the day after plating and every 3-4 days after plating.
        8. Once the SCs reach 70-80% confluence, repeat steps 1.2.3.1 to 1.2.3.6. The SCs are now at passage 2.
    4. Transduce the SCs once reaching 50% confluence, with a lentiviral vector encoding GFP in D10/3M medium overnight at a multiplicity of infection of 30 as previously described [44,45].
    5. Replenish with fresh D10/3M medium the next day and every 3-4 days after plating.
    6. Once the GFP-SCs reach 70-80% confluence, repeat steps 1.2.3.1 to 1.2.3.5 but resuspend the GFP-SCs in 6 mL of D10 medium instead. Dispense 15 µL of GFP-SC suspension into a 1.5 mL centrifuge tube and add 15 µL of 0.4% trypan blue solution. Flick the centrifuge tube gently and dispense 10 µL of the mixture into a chamber of the cell-counting slide.
        1. Place the slide into an automated cell counter and determine the cell density.
    7. Centrifuge the cells at 370 x g for 5 min at 4 ^{o}C. Remove the supernatant, resuspend with 1.5 mL of freezing medium for every 3x10^{6} GFP-SCs. Dispense 1.5 mL of GFP-SC suspension into a cryogenic vial, and freeze at -80^{o}C for at least 24 h before moving to liquid nitrogen for storage.
    NOTE: Freezing medium: 25% FBS and 8% dimethyl sulfoxide in DMEM.
    8. Thaw GFP-SCs one week before surgery.
        1. Add 10 mL D10 medium into a 50 mL centrifuge tube.
        2. Thaw vials of frozen GFP-SCs in water bath at 37 ^{o}C until partially thawed.
        3. Remove the GFP-SC suspension from the cryogenic vial and place it into the 50 mL centrifuge tube prepared in 1.2.8.1. Gently pipet the solution up and down repeatedly.
        4. Centrifuge the cells at 370 x g for 5 min at 4 ^{o}C. Remove the supernatant and resuspend the cells in 4 mL D10/3M medium for each vial thawed.
        5. Dispense 2 mL of GFP-SC suspension and 5 mL of D10/3M medium into a 10-cm Petri dish; each vial should result in 2 Petri dishes. The GFP-SCs are defined as passage 3.

NOTE: A 10-cm Petri dish usually yields around $8x10^6$ GFP-SCs after one week.

9. On the day of the surgery, repeat step 1.2.6. Use the cell density calculated in step 1.2.10.
10. Dispense aliquots of $3x10^6$ GFP-SCs into sterile 1.5 mL centrifuge tubes based on the cell density calculated from step 1.2.9. Centrifuge the GFP-SC aliquots at 370 x g for 5 min at 4 ^oC and remove the supernatant. Add 1 mL of DMEM/F12 medium to each aliquot and centrifuge at 370 x g.
    NOTE: Each animal receives $3x10^6$ GFP-SCs. Medium with serum is used with GFP-SCs until this step.
11. Keep GFP-SCs on ice until the time of transplantation.

2. Complete Transection at Thoracic Level 8 (T8)

1. **Animal preparation for surgery**
    1. Anesthetize a female Fischer rat (180-200 g) with an intraperitoneal injection of ketamine (60 mg/kg body weight) and xylazine (5 mg/kg body weight). Monitor the depth of anesthesia before proceeding to the next step by checking toe pinching and eye blinking responses.
    2. Shave the back of the rat with an electric clipper and wipe the skin with 70% ethanol. Apply ophthalmic lubricant to both eyes. Transfer the animal to the surgery table on a heating pad to maintain body temperature at about 37 ^oC. Place the animal on a roll so the vertebrae are easy to access.
        NOTE: The roll can be made from paper towel or 2 in x2 in gauze that can be sterilized. Larger rolls are recommended when T7-T9 needs to be laid flat on top of the roll.

2. **T7 to T9 laminectomy**
    1. Locate the landmark for the T9-T11 spinous processes.
        NOTE: The gaps between T9, T10, and T11 are small compared to other segments in the region. After placing the rat on the roll, T9-T11 spinous processes will feel like a small triangular bump through the skin.
    2. Make a 4 to 5 cm midline incision of the skin using a #10 blade from T4 to T11. Make a small incision in the superficial fat layer with curved scissors with blunt ends to dissect the fat.
        NOTE: Other types of instruments can be used to separate the fat but curved scissors with blunt ends enable the most effective and least invasive method for fat separation.
    3. Locate the T7 to T9 spinous processes by using the back of the #10 blade. Hold the muscle at about T4 with blunt forceps and make an incision in the muscle along each side of the vertebrae from T6-T10. Make the cut as close to the vertebrae as possible to make a clean opening and cause minimal injury to the animal.
    4. Isolate each individual spinous process from T7 to T9 by cutting the muscles and ligaments between T6 and T7, T7 and T8, T8 and T9, and T9 and T10 with a #10 blade.
    5. Use a rongeur to remove any muscle and ligaments on the laminae from T7 to T9. Remove the muscles and ligaments as laterally as possible until the gaps between the transverse processes from T7 to T9 are visible.
    6. Remove the T9 spinous process with a rongeur. Hold the T8 spinous process with blunt forceps and lift gently to elevate the T9 lamina at the small opening between the T9 and T10 processes.
    7. Remove the lamina starting from the opening and moving rostrally with a rongeur at T9. Remove the lamina laterally as much as possible; the dorsal roots should be visible after the laminectomy.
    8. Once the lamina is removed, repeat the process for T8 and T7.
        NOTE: During the laminectomy, use absorption spears to remove blood while continuing with the laminectomy. If excess bleeding occurs, place a compressed sponge at the bleeding site and add saline to help with blood clotting.

3. **Transection at T8**
    1. Place the retractor around T7 to T9. Once all the laminae are removed, make sure that the gaps between the transverse processes are visible, especially at T8. Also examine the bones along the length on both sides between T7 to T9 to ensure there are no bone fragments protruding outward.
        NOTE: This gap at T8 is important for performing the complete transection. The vertebrae along the length should be removed for easier conduit insertion.
    2. Cut the dorsal and ventral roots above and below T8 using angled spring scissors. Add saline to the spinal cord and wait for the blood to clot.
        NOTE: This step is important for proper conduit insertion.
    3. Place the angled spring scissors above the spinal cord in the gaps between the transverse processes at T8 and make one cut to completely sever the spinal cord. Place a small piece of compressed foam into the resulting 2-2.5 mm gap and add saline to the area immediately.

3. Conduit insertion

1. While waiting for the severed cord stumps to reach hemostasis, take out a conduit from 1x PBS. Cut the absorption triangles into thin long pieces and place them into the conduit to remove excess PBS. Check that the pre-cut windows are open.
2. Remove saline and blood using thin long pieces of absorption triangles. Lift the two stumps of spinal cord using a microspatula to ensure good separation. Gently lift the rostral stump with the microspatula and slip the conduit over the stump with the windows facing oneself. Ensure that the entire stump is inserted and there is no excess bleeding into the conduit.
3. Gently lift the caudal stump and slip the other end of the conduit over the stump. Make sure that the entire stump is inserted and the windows are on the dorsal surface (**Figure 2A**).

4. Schwann Cell/ Injectable Matrix (see Table of Materials) Preparation and Injection

1. While waiting for the severed cord to reach hemostasis, remove the medium above the GFP-SC pellet (prepared in step 1.2). Resuspend the GFP-SCs in 10 µl of cold DMEM/F12. Add 10 µl of cold injectable gel to the cell suspension and mix well by repeated pipetting. Keep the GFP-SC/DMEM/F12/injectable matrix mixture on ice until conduit insertion is completed.
2. After ensuring that the windows on the dorsal surface are open (**Figure 2A**), inject 20 µl of the GFP-SC/DMEM/F12/injectable matrix mixture into the conduit through one of the pre-cut windows[46] using a western blot loading tip and a micropipette. If the SC/DMEM/F12/injectable matrix mixture should overfill the conduit, remove the excess with absorption triangles. Close the windows after injection; suturing is not needed (**Figure 2B**).

5. Wound Closure and Postoperative Care

1. Suture the muscle layers and the superficial fat layers. Clean the skin with 70% ethanol and then staple (*e.g.,* using wound clips) the skin shut. Keep the animals in a thermally regulated incubator until they regain consciousness. Transfer the animals back into the cages. Provide water with a long feeding tube and place food pellets on the bedding for easy access.
2. Administer Buprenorphine (0.1 mg/kg body weight) twice a day for 3 days subcutaneously starting immediately after surgery to reduce pain. Inject gentamycin (5 mg/kg body weight) subcutaneously once a day for 7 days immediately after surgery to prevent and reduce infection. Inject 10 mL of lactated Ringers solution subcutaneously twice a day for 7 days for hydration.
3. Empty bladders manually twice a day until bladder function returns. If bladder infection occurs, then administer 10 mL saline subcutaneously until urine becomes clear. If there is no improvement in two days, inject gentamycin (5 mg/kg body weight, once daily) subcutaneously until the urine becomes clear.

Representative Results

The goal of using this surgical technique is to evaluate the use of a structured conduit and injectable matrix that maximizes SC function after transplantation into completed transected spinal cords. Three weeks after transplantation, the animals are perfused with 4% paraformaldehyde and the spinal columns are grossly dissected and fixed in the same fixative for another 24 h. The spinal cord is then dissected and the samples for cryostat sagittal sections are placed into a 30% sucrose solution for cryoprotection. 1-mm thick cross sections isolated from the middle of the SC bridge of another set of dissected spinal cords are placed into glutaraldehyde fixative to process for plastic sections. The samples are subjected to a schedule for electron microscopic preparation as detailed by Bates *et al.*[47]. Cryostat sagittal sections were immunostained with primary antibody against GFP, glial fibrillary acidic protein (GFAP), heavy chain neurofilament (RT97), and medium chain neurofilament (NF) followed by secondary antibodies including goat-anti-chicken-488, goat-anti-rabbit-546, goat-anti-mouse-647, and goat-anti-rabbit 647, respectively. GFP-SCs were distributed evenly along and within the conduit (**Figure 3A**; inner walls indicated by yellow lines). Axon regeneration is observed (**Figure 3B**) and is closely associated with the presence of GFP-SCs (**Figure 3A** and **Figure 3C**) indicating that this technique is successful in providing a SC bridge within a structured conduit and in promoting axon regeneration along the bridge between the rostral and caudal stumps. Blood vessels and myelinated axons were also found in the center of the SC bridge (**Figure 3D**). More details of the effect of SCs with electrospun PVDF-TrFE fibrous conduits on axon regeneration can be found in our recent published work[31].

Other outcome measures can be performed including: quantifying axon regeneration in sagittal sections by the line-transect-method described in our recent work[31] and quantifying the number of myelinated axons and vessels in plastic cross sections. Samples prepared for plastic sections can be further sectioned for transmission electron microscopy to quantify the number of unmyelinated axons. The cryoprotected spinal cord samples can also be cross-sectioned instead of sagittally sectioned and immunostained to quantify axon regeneration and the presence of GFP-SCs. Behavioral tests can also be performed on the animals at appropriate intervals while the animals are being maintained.

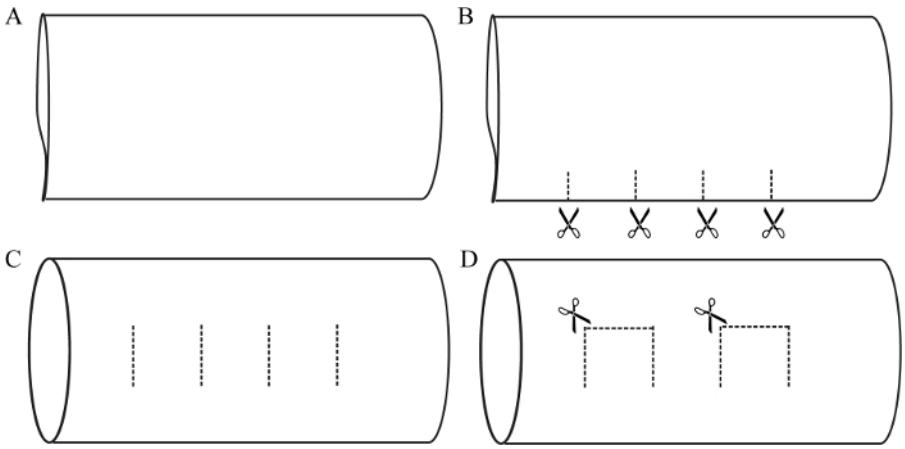

Figure 1: Window preparation in the conduit. Fold one side along the longitudinal axis of the 5 mm conduit (**A**). Cut four incisions about 0.4 mm long and at least 1 mm from the openings of the conduit (**B**). Each incision is about 1 mm apart. By unfolding the conduit, the 4 parallel cuts are observed (**C**). Cut between the two incisions along the rostral-caudal axis to create a window that can be opened by lifting the flap. Please click here to view a larger version of this figure.

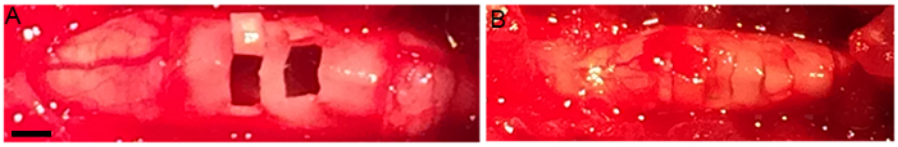

Figure 2: Window closing after SC/DMEM/F12/matrix injection. Windows are opened by folding back each flap after placing the conduit between rostral and caudal stumps (**A**). Windows are closed after injection (**B**). Scale bar = 1 mm. Please click here to view a larger version of this figure.

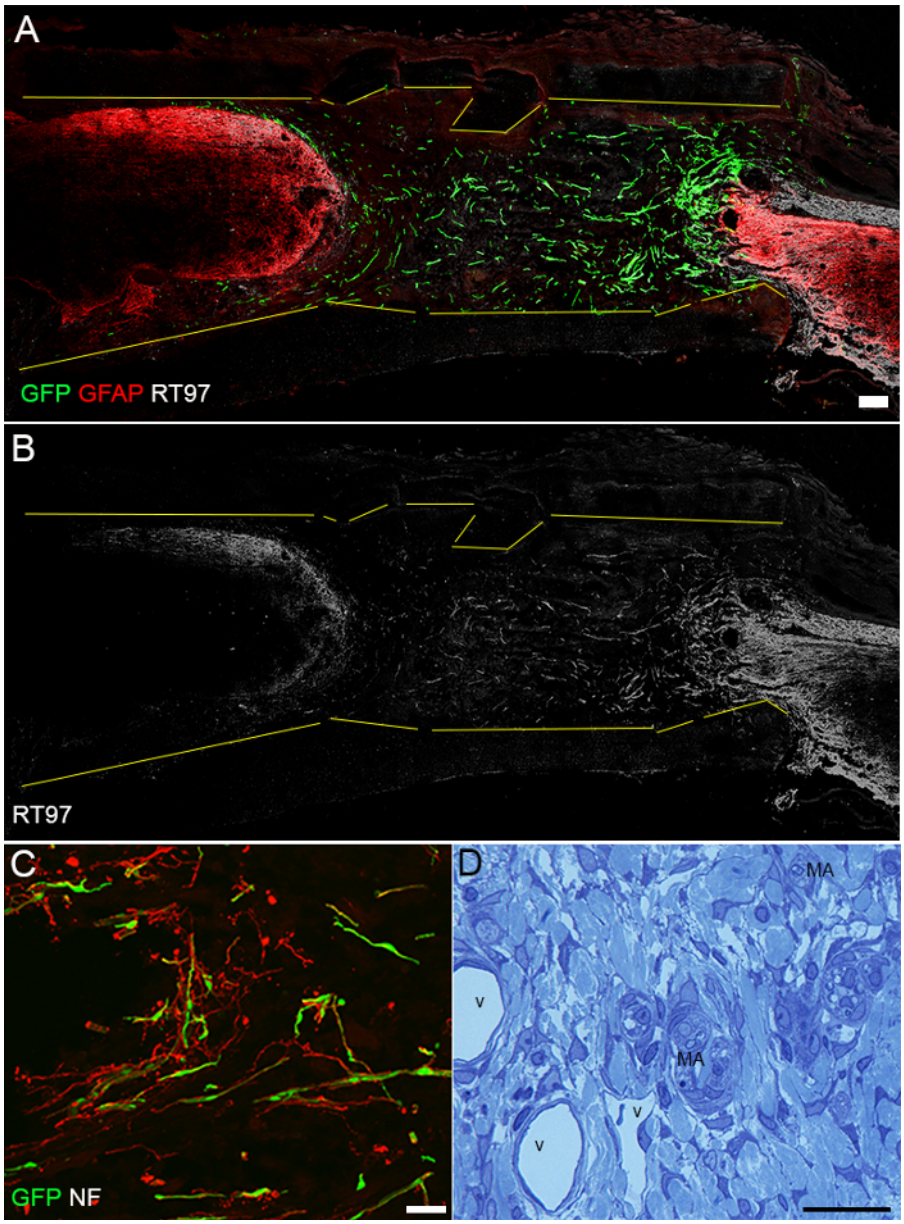

Figure 3: Confocal fluorescent images of sagittal sections and a bright field image of a plastic cross-section from spinal cords transplanted with GFP-SCs in aligned fibrous PVDF-TrFE conduits. Overview of the SC bridge within the conduits where the inner walls are indicated by the yellow lines and immunostained for GFP and glial fibrillary acidic protein (GFAP) to visualize transplanted SCs and host spinal cord astrocytes, respectively. Regenerated axons were labeled with RT97 (heavy-chain neurofilament) (**A,B**) and medium-chain neurofilament (NF, **C**) antibodies. Axons are not as visible in (**A**) due to their close association with GFP-SCs as is observed in the mid-conduit region in (**C**) with higher magnification. Axons are not visible in the rostral stump due to the incomplete scan in the depth of the tissue section when imaging by confocal microscopy. Blood vessels (labeled as v in **D**) and myelinated axons (labeled as MA in **D**) were both observed in the mid-conduit region in a plastic section. Magnifications and scale bars: A,B (10x, 200 µm); C (20x, 100 µm); D (63x, 25 µm). Please click here to view a larger version of this figure.

Discussion

The most critical step in creating an effective transection model is severing the spinal cord in one or two cuts. A 2-2.5 mm gap between the rostral and caudal spinal cord stumps should be present at the transection site. The three most likely reasons for such a gap not appearing are (1) the dorsal/ventral roots were not removed properly, (2) the ventral dura was not removed adequately, and/or (3) the animal was not positioned properly on the roll placed beneath her.

To perform an effective conduit insertion between the stumps: the (1) diameter of the conduit should be tailored for the specific species and age of the animal used in the experiment; (2) laminae must be removed laterally enough until the gap between the transverse processes can be visualized; (3) roots must be removed and (4) conduit insertion between the stumps should be accomplished on the first try. If multiple attempts are needed, this may cause edema in the stumps, further complicating the task of conduit insertion between the stumps and causing additional injury.

To perform effective GFP-SC/DMEM/F12/injectable matrix introduction into the conduit, ensure that: (1) the spinal cord is not bleeding before conduit insertion between the stumps. If there is fluid in the conduit after insertion between the stumps, use a small piece of absorption spear to remove it via the pre-cut windows. (2) Ensure that the conduit is slipped onto both spinal cord stumps well and (3) that the dorsal pre-cut windows are open. The injection of 20 µl of the SC/injectable matrix mixture should be more than enough to fill the conduit. Overflow from the pre-cut windows is a good gauge for effective transplantation.

The limitations of this procedure are: (1) no restoration of the dura, (2) no control over the movement of the conduit/SC transplant after completion of the surgery, and (3) no alternative method if there is leakage while injecting the SC/injectable matrix mixture.

The advantage of this procedure is the combination of the use of both a structured conduit with an injectable matrix. Any conduit made with a material of choice that is pliable to be inserted between the stumps can be used in this surgical procedure. Any injectable matrix of choice can also be used in this procedure; a temperature-sensitive gel is preferable due to its ability to gel *in situ* and contour to the shape of the stump creating a seamless interface. Conduits with a more complex interior structure can be used instead of a hollow interior. Drugs, growth factors, small molecules, or any cell type can be incorporated into the structured conduit or the injectable matrix or both to enhance transplant survival, provide neural protection immediately after injury, reduce inflammation, promote axon regeneration, and promote angiogenesis.

Disclosures

The authors have nothing to disclose.

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