

Video Article

Production and Use of Lentivirus to Selectively Transduce Primary Oligodendrocyte Precursor Cells for *In Vitro* Myelination Assays

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Abstract

Myelination is a complex process that involves both neurons and the myelin forming glial cells, oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). We use an *in vitro* myelination assay, an established model for studying CNS myelination *in vitro*. To do this, oligodendrocyte precursor cells (OPCs) are added to the purified primary rodent dorsal root ganglion (DRG) neurons to form myelinating co-cultures. In order to specifically interrogate the roles that particular proteins expressed by oligodendrocytes exert upon myelination we have developed protocols that selectively transduce OPCs using the lentivirus overexpressing wild type, constitutively active or dominant negative proteins before being seeded onto the DRG neurons. This allows us to specifically interrogate the roles of these oligodendroglial proteins in regulating myelination. The protocols can also be applied in the study of other cell types, thus providing an approach that allows selective manipulation of proteins expressed by a desired cell type, such as oligodendrocytes for the targeted study of signaling and compensation mechanisms. In conclusion, combining the *in vitro* myelination assay with lentiviral infected OPCs provides a strategic tool for the analysis of molecular mechanisms involved in myelination.

Video Link

The video component of this article can be found at <https://www.jove.com/video/52179/>

Introduction

Myelination of axons is crucial for the fast and efficient transmission of action potentials in both the central and peripheral nervous systems. Specialized cells, Schwann cells in the peripheral nervous system and oligodendrocytes in the central nervous system, wrap around and ensheath axons in myelin, effectively insulating the nerve and facilitating saltatory conduction¹. The process of myelination can be studied *in vitro* using retinal ganglion neurons², engineered nanofibers³, or dorsal root ganglion neurons co-cultured with either Schwann cells⁴ or oligodendrocytes⁵⁻⁷. The *in vitro* myelination assay is an established model for studying nervous system myelination and it replicates many of the fundamental processes that occur during myelination *in vivo*⁵⁻⁸. The assay involves the coculture of purified populations of Dorsal Root Ganglion (DRG) neurons, with OPCs (for CNS myelination) or Schwann cells (for PNS myelination). Under specific conditions these myelinating cells ensheath DRG axons in the ordered, ultra structurally verified, multi-lamellar sheet of insulating plasma membrane that express the same complement of myelin specific proteins present *in vivo*.

The most commonly used cell model of studying CNS myelination *in vitro* is the co-cultures of DRG neurons and OPCs, which have been successfully used to study the effect that exogenous factors such as the neurotrophins exert on CNS myelination *in vitro*^{5,6}. Exogenous factors such as growth factors or small molecule pharmacological inhibitors have been widely used to study the role of signaling pathways in myelination using the DRG-OPC coculture model^{7,9}. However, in the mixed co-culture settings that contain both the neurons and oligodendrocytes, it remained formally possible that either the growth factors or the pharmacological inhibitors could have exerted effects upon both the DRG neurons and oligodendrocytes (OL). This does offer the ability to specifically dissect the roles that the proteins expressed only by DRGs or oligodendroglia exerts upon myelination using this dual cell system. To unequivocally confirm that the signaling pathway in oligodendroglial directly regulates myelination, lentiviral transduction of OPCs, prior to seeding onto DRG neurons for the *in vitro* myelination assay, has proven to be an elegant way to overexpress both wild-type and mutant proteins, as well as knockdown expression of constitutively expressed proteins by oligodendrocytes. Thus this approach offers an avenue to specifically interrogate and manipulate signaling pathways within oligodendrocytes for studying myelination^{9,10}.

In this paper, we report methods that we have developed to overexpress a protein of interest selectively in oligodendrocytes via a lentiviral approach for studying myelination *in vitro*. The technique begins with the generation of expression vectors containing the gene of interest, be it in a wild type, constitutively active or dominant negative form which are then subsequently cloned into the pENTR vector (pENTR L1-L2 pENTR4IRES2GFP). This vector (containing the gene of interest), the CMV promoter donor (pENTR L4-R1 pENTR-pDNOR-CMV) and the 2K7 lentivector are combined in an enzyme reaction to produce a 2K7 vector containing CMV promoter, the gene of interest, an internal ribosomal

entry site and GFP (**Figure 1**). This Gateway cloned 2K7 construct combined with the PMD2.G virus envelope and the pBR8.91 virus package can be co-transfected into HEK293T cells to generate lentivirus that can subsequently be used to transduce OPCs. Once infected with the lentivirus the OPCs express a high level of the protein of interest. These OPCs can then be seeded onto DRG neuron cultures and the effect that expression of high levels of the desired protein exerts on myelination can be interrogated. The co-cultures are assessed for myelin protein expression by western blot analysis and visualized for the formation of myelinated axonal segments by immunocytochemistry.

Protocol

NOTE: All animals used for this study were of mixed sex and bred at the Animal Facilities of the Department of Anatomy & Pathology and The Florey Institute of Neuroscience and Mental Health Research at the University of Melbourne. All animal procedures were approved by Animal Experimentation Ethics Committees at the University of Melbourne.

1. Cloning of 2K7 Lentivector

- Before cloning the gene of interest into the 2K7 lentiviral vector, subclone the gene into the pENTR vector (3637 bp, Kanamycin resistant) using standard molecular techniques¹¹. Use the EcoRI and SacII restriction sites for the subcloning.
- Amplification of the 2K7 Lentivector
 - Transform 2K7 lentivector DNA by gently mixing 100 ng of plasmid DNA with competent cells and incubate on ice for 30 min.
 - Heat shock DNA/ competent cells mix at 42 °C for 90 sec and plate them on LB-Agar plates containing both ampicillin (100 µg/ml) and chloramphenicol (15 µg/ml) at 37 °C for 16-18 hr.
NOTE: Chloramphenicol is used to prevent recombination between the long terminal repeats.
 - The next day, grow selected bacterial clones in LB media containing both ampicillin (100 µg/ml) and chloramphenicol (15 µg/ml) at 37 °C for 16-18 hr. Extract the DNA using a commercial plasmid DNA Maxiprep kit as per manufacturer's instructions.
- Sub-cloning from pENTR vector to 2K7 Lentivector (**Figure 1**)

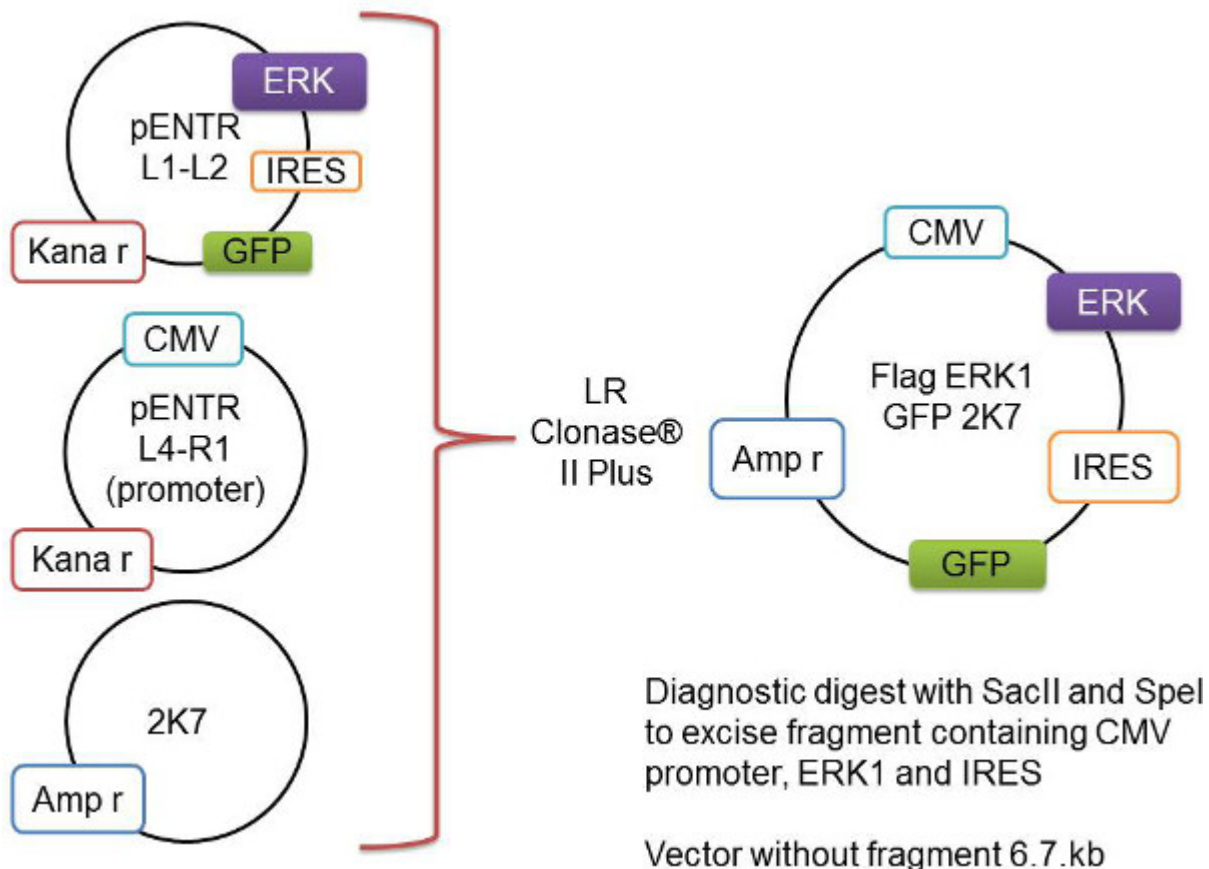


Figure 1: Schematic representation of the Gateway recombination process. The gene of interest, here represented as Flag-Erk1, is cloned into the pENTR L1-L2 vector. This is added to the pENTR L4-R1 vector containing the CMV promoter and the backbone 2K7 vector. These three vectors are recombined by the LR Clonase II Plus enzyme to insert the gene-of-interest and the promoter into the virus-ready 2K7 vector.

- Add the following to a 1.5 ml tube and mix gently:
 - L4-R1 pENTR-pDNOR-CMV (promoter) (60 bng) 1-2.5 µl
 - L1-L2 pENTR4IRES2GFP (with gene of interest) (80 bng) 1-2.5 µl
 - K7 lentivector (80 ng/µl) 1 µl
 - TE-buffer, pH 8-5 µl

Total 8 μ l

2. Remove clonase enzyme mix from -80 °C and thaw on ice for 2 min. Ensure that this enzyme is a fresh aliquot from -80 °C as the enzyme has substantially reduced cloning efficiency with repeated freeze-thaw cycles
3. Add 2 μ l of enzyme per reaction and mix well via vortex, and incubate the clonase reaction mixture at 23-25 °C for 6-24 hr.
4. Add 1 μ l of proteinase K solution (supplied with enzyme kit) to the clonase reaction mixture and incubate for 10 min at 37 °C.
5. Transform clonase reaction mixture into competent cells and grow the select colonies in LB media containing ampicillin (100 μ g/ml) at 37 °C for 16-18 hr.
6. Extract and purify DNA using a commercial plasmid DNA Miniprep kit as per manufacturer's instructions.
7. Confirm the DNA via digestion using restriction enzymes Spe I and Sac II and an appropriate buffer as per manufacturer's instruction to remove the fragment containing the promoter and gene of interest.
NOTE: This step is critical to check if the subcloned DNA has the correct insert gene of interest with the right vector backbone. The size of the vector itself without inserted fragment is 6.7 kb. The size of the released inserted fragment includes both the promoter and the insert gene of interest. Calculate the expected fragment sizes from the digest for the specific gene via a DNA sequence editing program, e.g., APE – A Plasmid Editor.
8. Check the sizes of both DNA vector backbone and the released fragment by running a 1% agarose gel in 1x TAE buffer (see Table of stock solutions) at 100 V.
9. Amplify the confirmed DNA by growing bacteria in 500 ml of LB media containing ampicillin (100 μ g/ml) at 37 °C for 16-18 hr.
10. Extract and purify DNA using a commercial plasmid DNA Maxiprep kit as per manufacturer's instructions.
NOTE: Maxiprep typically generates sufficient amount of DNA required for viral production.

4. Repeat steps 1.3.9-1.3.10 to amplify the following DNAs for lentiviral preparations: Envelope vector (PMD2.G, 6.1 kb), Package vector (pBR8.91, 12.5 kb), and an empty lentiviral vector as a control (GFP-CMV-2K7, 8.7 kb).

2. 2K7 Virus Production

NOTE: Day 1:

1. On the day of transfection, plate 32 million HEK293 T cells in T175 flask containing 25 ml HEK293 T cell media (see the Table of stock solutions). Alternatively, plate 16 million cells the day before the transfection if time runs tight on the day of transfection.
NOTE: Transfection can be equally successful by plating cells on the day of transfection or prior to the day. Using either of the two alternatives, the critical point here is to make sure cells be stuck down on culture dish surface prior to transfection.

NOTE: Day 2:

2. Transfection
 1. Prior to transfection, dilute DNA to 1 μ g/ μ l in TE Buffer that contain 10 mM Tris pH 8, 1 mM EDTA pH 8 in deionized water.
 2. In a 50 ml tube, prepare a master mix (**Table 1**) for transfection in the T175 flask. Add DNA to pre-warmed Dulbecco's Modified Eagle Medium (DMEM) and mix well by vortex, then add sterile polyethylenimine (PEI) (see the Table of stock solutions) to avoid premature precipitation.

Vector	Concentration	Volume
pMDG.2	1 μ g/ μ l	5 μ l
pBR8.91	1 μ g/ μ l	15 μ l
2K7 vector with GFP + gene of interest	1 μ g/ μ l	22 μ l
Sterile Polyethylenimine (PEI)	1 g/L	500 μ l
DMEM		2,100 μ l

Table 1: Preparing transfection mix for 2K7 Virus.

3. Mix well by inverting the tube 3-4x and incubate for 15 min at room temperature (RT).
4. Perform a full media change on HEK293T cells. Aspirate off the culture media from cells completely and feed with pre-warmed HEK293T cell culture media (25 ml per T175 flask).
5. Add the transfection mixture (DNA/PEI mixture) drop wise to the monolayer cells. Move gently to mix well and incubate transfected cells at 37 °C, 5% CO₂, overnight.

NOTE: Day 3:

6. To ensure the transfection is successful, check GFP expression 24 hr post-transfection via a fluorescent microscopy.
NOTE: Over 50% of cells expressing GFP typically indicates a good transfection.

NOTE: Day 4:

3. At the 48 hr post-transfection, collect the viral supernatant and replace with fresh HEK293 T media (25 ml per T175 flask).
 1. Centrifuge the viral supernatant at 1,140 x g for 10 min at 4 °C to clear up cell debris from the supernatant. Transfer cleared supernatant to 50 ml tube and store at 4 °C.
NOTE: Day 5:
4. At 72 hr post-transfection, collect the second batch of viral supernatant. Repeat step 2.3.1 and pool 48 and 72 hr cleared supernatants.
5. To concentrate the virus, centrifuge viral supernatant at 170,000 x g for 90 min at 4 °C using 30 ml ultracentrifuge tubes.
6. Discard the supernatant and repeat step 2.6 until all the cleared supernatant has been centrifuged leaving an (invisible) pellet of virus plus precipitated PEI in the base of the tube.
7. To resuspend virus, add 500 μ l SATO media (see Table of stock solutions) to the ultracentrifuge tubes. Vortex for 30 sec and scrape the base of the tube with a pipette tip to mechanically loosen the virus. Repeat this step 6x in order to loose viral pellet.

8. Pool the resuspended virus into microcentrifuge tubes and spin very briefly to remove insoluble PEI. Filter the supernatant through a 0.45 μ m filter to remove proteins.
9. Aliquot the virus into 20 μ l, 50 μ l, and 100 μ l aliquots and store at -80 °C.

3. Viral Titer Determination in HEK293T Cells

1. To check the expression of protein of interest and to determine the optimal viral concentration for experiments, add a series of serial dilutions of viral stock (e.g., 0, 5, 10, 20, 40, 80 μ l) to transduce HEK293T cells plated in 6-well plates, and culture for 24 hr at 37 °C, 5% CO₂. This protocol typically generates virus that can be used at concentrations ranging from 1:50 to 1:200 that achieve robust expression of the gene of interest.
2. 48 hr post viral transduction, lyse HEK293T cells in TNE buffer (see Table of stock solution) with protease inhibitors.
 1. Rinse wells twice with chilled DPBS and then add 150 μ l TNE buffer to each well.
 2. Lyse cells by pipetting up and down 5-10x. Transfer whole cell lysates to 1.5 ml microcentrifuge tube and incubate on ice for 15-30 min.
 3. Centrifuge the lysates at 4 °C for 30 min at maximum speed (20,000 x g) and transfer cleared supernatant to a fresh tube for protein determination by Bradford and subsequent western blot analysis.
3. Determine the level of expression of the protein of interest by standard western blot analysis while probing for antibodies against the protein itself and the fused tag (i.e., Flag). Use the viral dilution that yields >95% GFP+ cells and robust expression of protein of interest for experiments.

4. Isolation and Culture of DRGs (Figure 2 steps 1 & 2)

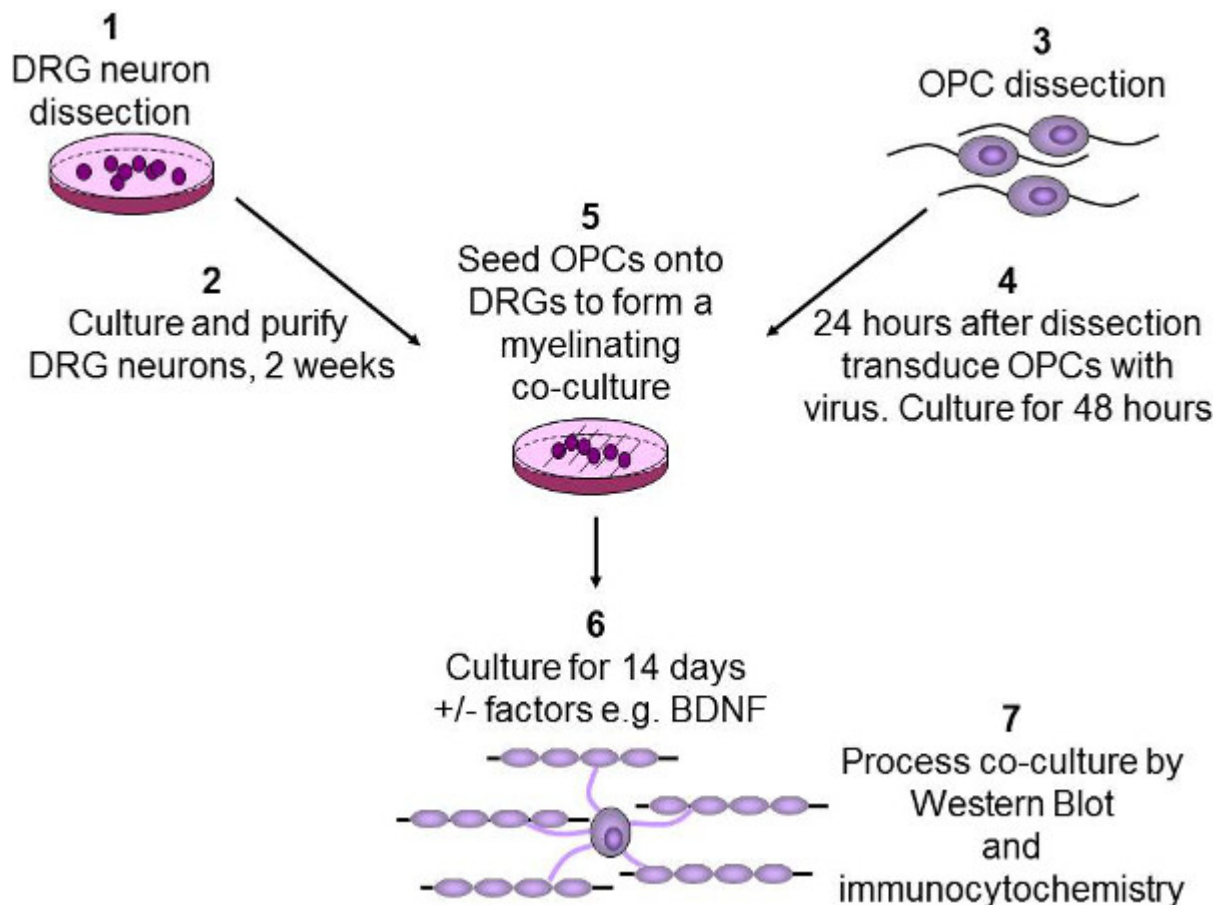


Figure 2: Schematic diagram of the *in vitro* myelination assay. DRG neurons are dissected from P2-3 rat pups, then purified and cultured over two weeks (1-2). OPCs are purified from P7-9 rat brains using immunopanning (3). OPCs are then infected with lentivirus and cultured for 48 hr (4). OPCs are then seeded onto DRGs, and any growth factors of interest such as BDNF are added (5). Co-cultures are then cultured for 2 weeks to allow OPCs to differentiate and myelinate the axons (6). Finally, co-cultures are either lysed for western blotting or fixed for immunocytochemistry (7).

NOTE: Day 1- 2 days prior to dissection:

1. Coat autoclaved 22 mm x 22 mm coverslips with Poly-L-ornithine (0.5 mg/ml) in a 6-well plate, and incubate at 37 °C overnight.
2. On the next day, coat coverslips again with Laminin (20 μ g/ml in MEM) at 37 °C overnight, aspirate off excess and dry for 20 minutes in tissue culture hood.

NOTE: Day 2: Dissection and isolation of DRGs:

3. Sacrifice P2-P3 rat pups 12x by cervical transection.
4. Remove the skin overlying the muscle from the back of the animal. Use vannas scissors to open vertebral foramen and use forceps to gently scoop out the spinal cord.
5. Pluck out DRGs that lie in between vertebral columns and cut off attached spinal nerve fibres, then place DRGs in a 33 mm Petri dish containing 3 ml L-15 media. It is usually possible to gather between 8 and 12 DRG from each side of the spinal cord
6. Transfer collected DRGs along with the L-15 media into a 15 ml tube, centrifuge at 180 x g for 5 min.
7. Aspirate the supernatant, add 2 ml 0.25% trypsin to DRG pellets and incubate at 37 °C for 30 min.
8. Add 5 ml of M1 medium (see Table of stock solution) to the DRG pellets to stop trypsin, and centrifuge at 180 x g for 3 min at RT.
9. Aspirate supernatant, re-suspend the pellet in 2 ml pre-warmed M1 medium (not with growth factors). Triturate the ganglia pellet by pipetting 50x or until the ganglia are dispersed. Centrifuge cell suspension at 180 x g for 3 min.
10. Resuspend dissociated neurons in M1 media and plate down in a 6-well plate at 5 ganglia per 100 µl per well.
11. To remove proliferating non-neuronal cells, after a minimum of 4 hr incubation to facilitate attachment, remove M1 media and feed neurons with M2 media (see Table of stock solution). Culture DRG neurons in the presence of NGF (100 ng/ml) to purify a culture of NGF-dependent TrkA-expressing DRGs. Alternatively, use BDNF (100 ng/ml) to purify BDNF-dependent TrkB-expressing DRGs.
12. Maintain neurons in M1 media (+NGF or BDNF at 100 ng/ml) alternating with antimitotic M2 media for 2 weeks as below.
13. Maintain medium M1 (+NGF or BDNF at 100 ng/ml) on days: 4-6, 8-10, 12-14 and M2 media (+NGF or BDNF at 100 ng/ml) with FDU and uridine on days 1 to 4, 6-8, and 10-12. A minimum of 3 cycles of M2 media purification is required.
14. Maintain DRGs in M1 alone for a further week after completing the 2 week antimitotic cycle. Change M1 media every 2-3 days.

5. Isolation and Culture of OPCs (Figure 2 step 3)

NOTE: Day 1- 2 days prior to dissection:

1. Coat 10 cm tissue culture plates with Poly-D-Lysine (PDL, 10 µg/ml in sterilized deionized water) at 4 °C overnight.
NOTE: Day 2: 1 day prior to dissection:
2. Wash PDL plates with sterilized deionized water for 3x. Allow to dry for ~6 hr in tissue culture hood. If not being used straight away, wrap and store for up to 4 weeks at 4 °C.
3. Prepare secondary antibody plates for immunopanning. For 1 brain dissection, prepare 2x IgG plates (for Ran2 antibody to remove astrocytes and O1 antibody to remove premyelinating oligodendrocytes), with 45 µl Goat α mouse IgG in DPBS (15 ml) per 10 cm Petri dish; 1x IgM plate for (O4 antibody for selecting oligodendrocyte precursor cells), 45 µl Goat α mouse IgM in DPBS (15 ml) per 10 cm Petri dish.
NOTE: Day 3: day of dissection:
4. Add 200 units of papain in 10 ml of papain buffer (Table 2), and warm up at 37 °C until the buffer turns clear.

	Concentration	for 250 ml	Final concentration
EBSS stock	10x	25 ml	1x
MgSO ₄	100 mM	2.5 ml	1 mM
Glucose	30%	3 ml	0.46%
EGTA	0.5 M	1 ml	2 mM
NaHCO ₃	1 M	6.5 ml	26 mM
Bring volume up to 250 ml with deionized water and filter sterilize			

Table 2: Preparation of papain buffer.

5. Wash all secondary antibody plates with DPBS for 3x.
6. Pour the Ran2 and O1 antibody onto the IgG plates and the O4 hybridoma to the IgM plate. Incubate all these primary antibody plates for over 2 hr at RT.
7. Dissect one brain from a P7 rat. Decapitate a pup with sharpened scissors and remove the skin overlying the skull with scissors.
 1. Cut around the skull from the occipital lobe, the temporal lobe and to the frontal lobe. Remove brain from skull using a forceps and gently transfer it to a 35-mm Petri dish with 1 ml DPBS.
 2. Dice the brain into small pieces roughly with scissors or sterile blade.
8. Filter pre-warmed papain buffer (10 ml) into a new 15 ml tube containing a few grains of L-cysteine, then add 200 µl DNAase (12,500 U/ml) to the filtered papain buffer.
9. Pour the papain buffer onto the diced brain tissues; incubate at 37 °C for 90 min.
10. Gently transfer dissociated brain tissues into 50 ml tube using a 25 ml pipette and allow to settle.
11. Remove papain buffer, add 2 ml Lo Ovo (Ovomucoid) to brain tissues and titrate 5-10x by pipetting to break up chunks of brain tissues, allow to settle and remove the top 2 ml of supernatant to a new tube.
12. Add another 2 ml Lo Ovo to brain tissues and repeat step 5.11 until no chunks of tissue remain. Titration can get increasingly aggressive.
13. Centrifuge dissociated cell suspension for 15 min at 200 x g. Aspirate off supernatant and resuspend cell pellet in 10 ml Hi Ovo and centrifuge for 15 min at 200 x g.
14. Wash first immunopanning plate (Ran 2 plate) with DPBS for 3x. Aspirate supernatant, resuspend cells in 10 ml panning buffer (Table 2) and pour on to the first immunopanning plate (Ran 2 plate). Incubate cells for 15 min at RT.
15. Wash the second immunopanning plate (O1 plate) with DPBS for 3x.
16. After the incubation on the first immunopanning plate, tip the cell suspension onto second immunopanning plate (O1 plate). Rinse any loose cells off the surface of the plate with 1-3 ml of panning buffer and transfer by pipette to the O1 plate. Incubate cells for 15 min at RT.
17. Wash the third immunopanning plate (O4 plate). This plate is the positive selection plate where the OPCs bind its surface. Wash the O4 plate with DPBS for 3x.

18. After the incubation on the second immunopanning plate, transfer the cells to the final immunopanning plate (O4 plate), incubate cells for 45 min at RT. This step will select O4+ OPCs.
19. Aspirate the supernatant from the last immunopanning plate (O4 plate) and rinse the plate with EBSS for 6x.
20. To remove OPCs off the plate, incubate cells with 5 ml of warm 0.05% Trypsin-EDTA diluted 1:10 with EBSS at 37 °C for 8 min.
21. Add 5 ml of 30% FBS (made in EBSS) to neutralize the trypsin. Remove cells off surface of the plate by pipetting for about 50x.
22. Transfer all cell suspension to a new tube and centrifuge for 15 min at 200 x g.
23. Discard supernatant and resuspend cell pellet in 1 ml pre-warmed SATO media, followed by cell counting. Dissection of one brain can yield 1.5-2 million OPCs.
24. Plate cells onto dry PDL coated plates at a density between 1×10^5 and 5×10^5 per 10 cm plate with in SATO media (10 ml) that contain ciliary neurotrophic factor (CNTF, 10 ng/ml), platelet derived growth factor (PDGF, 10 ng/ml), neurotrophin 3 (NT3, 1 ng/ml), and forskolin ($4.2 \mu\text{g/ml}$)^{2,12}. Culture OPCs at 37 °C, 8% CO₂.

6. Transducing OPCs

1. Culture primary OPCs in SATO media (10ml/10cm plate) with CNTF (10 ng/ml), PDGF (10 ng/ml), NT3 (1 ng/ml), and forskolin ($4.2 \mu\text{g/ml}$) at 37 °C, 8% CO₂ for 24 hr after dissection.
2. Completely aspirate off the OPC culture media, feed cells with freshly made SATO media (10 ml) with growth factors (see above in step 6.1).
3. Add virus to the OPCs to the optimal concentration determined by step 3 (**Figure 2**, step 4), culture OPCs for a further 48 hr.

7. OPC Seeding for Myelinating Co-cultures (Figure 2, steps 5 and 6)

1. To remove OPCs off the surface, first rinse OPC plates with 8 ml EBSS twice, then incubate cells with 5 ml of warm 0.05% Trypsin-EDTA diluted 1:10 with EBSS at 37 °C for 2 min.
2. Neutralize the trypsin with 30% FBS in EBSS (5 ml) and remove cells from plate by pipetting. Transfer cell suspension to a 15 ml tube, centrifuge at 180 x g for 15 min at RT.
3. Aspirate off the supernatant and resuspend cell pellet in 1 ml pre-warmed SATO media followed by cell count.
4. Prior to the OPC seeding, completely aspirate media from the DRG culture plate. Gently seed 200,000 OPCs drop wise on to the DRG neurons as previously described⁴⁻⁶.
NOTE: The total OPC seeding volume must be less than 200 μl per 22 mm coverslip.
5. Leave cells to settle without moving the plate for 10 min in the tissue culture hood, then gently top up with 1 ml pre-warmed SATO media per well.
6. Replate the remaining sister OPCs with SATO media with growth factors (see step 6.1). Use these sister OPCs to verify expression of the protein of interest.
7. After 24 hr, replace the SATO media with the co-culture media (2 ml/well) containing SATO media (no factors) and neurobasal (v/v) with 1% B27. Maintain the co-cultures for 14 days with media change every 2-3 days.
8. Assess the co-cultures for myelin protein expression by western blot analysis and visualize for the formation of myelinated axonal segments by double-immunostaining with antibodies against myelin basic protein markers and neuronal markers⁴⁻⁶.

Representative Results

The flag-tagged extracellular signal-related kinase 1(Flag-Erk1) construct used for lentivirus production is verified by restriction enzyme digest of the constructs used, including both the 2K7 constructs and the packaging and accessory constructs required for virus production (**Figure 3**).

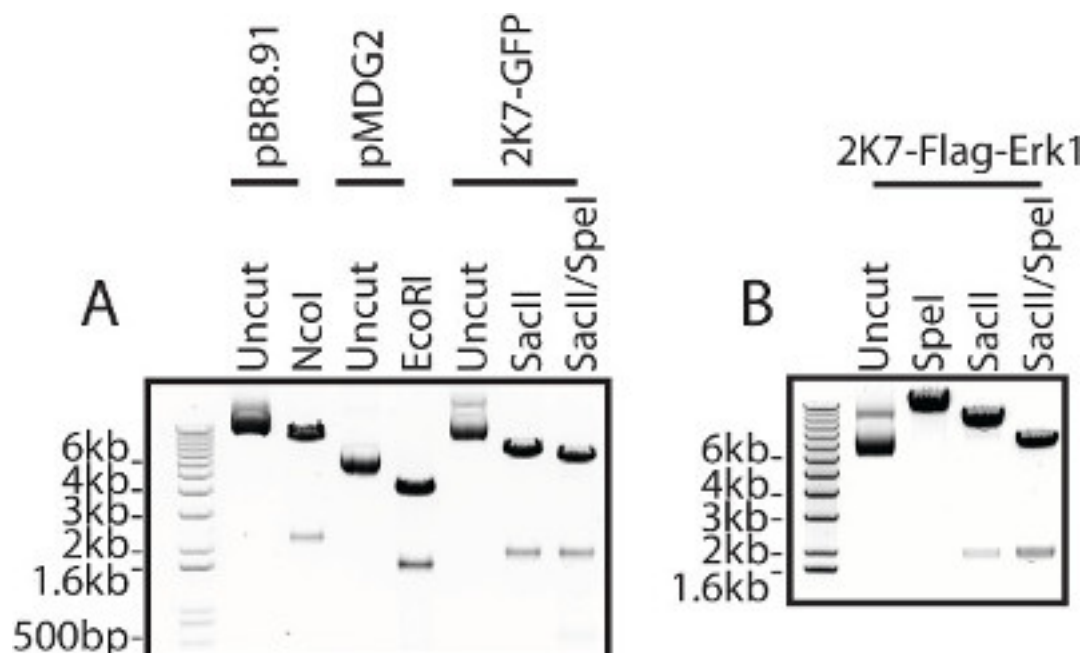


Figure 3: DNA construct verification. All DNA constructs required for lentivirus production were purified from Stbl3 bacteria and verified by restriction enzyme digest. Accessory and packaging vectors were digested with NcoI and EcoRI, as indicated, and compared to uncut plasmid (A). 2K7-GFP was digested with SpeI and SacII (A). 2K7-Flag-Erk1 DNA was verified by digestion with either SpeI or SacII alone, or by double digest (B). Banding patterns were compared to expected patterns generated from virtual digestion of the construct sequences using ApE software.

To determine the optimum dilution to use on OPCs, the Erk 1 lentivirus was titrated in HEK293T cells (Figure 4). This was done by adding a range of viral dilutions to HEK293T cells (Figures 4A, 4C, and 4D) or to OPCs (Figure 4B). Expression levels of the gene-of-interest increase with time (Figure 4D). [Please click here to view a larger version of this figure.](#)

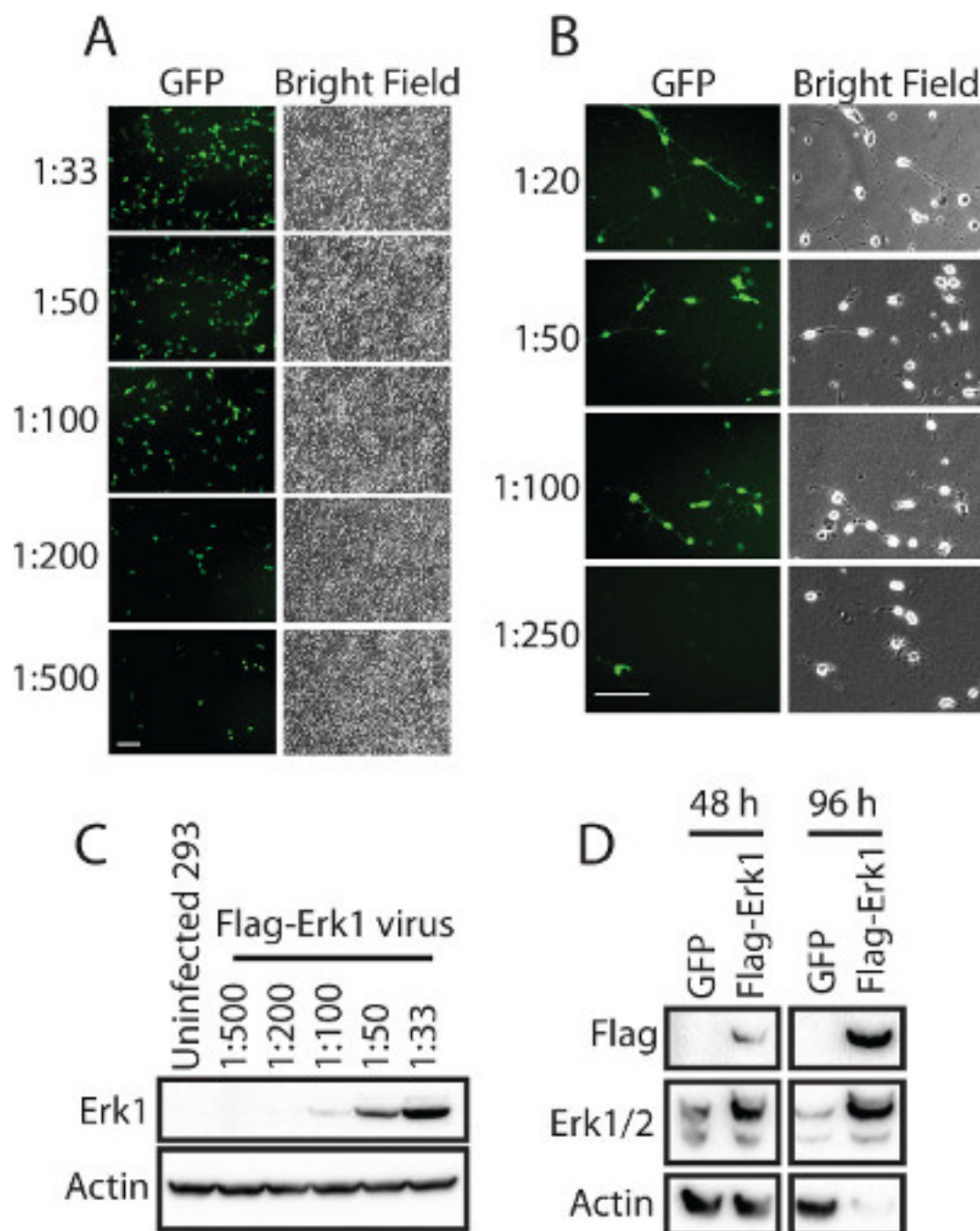


Figure 4: Titration of Flag-Erk1 virus in HEK293T cells and OPCs. Flag-Erk1 virus was titrated in HEK293T cells (**A**, **C**, **D**) or OPCs (**B**). HEK293T cells (**A**) or OPCs (**B**) were imaged for GFP expression 48 hr after infection with Flag-Erk1 virus. HEK293T cell lysates were probed for the presence of Erk1 or total Erk1/2 after 48 hr of infection (**C**). HEK293T cell lysates were probed for the presence of Flag, Erk1/2 and Actin as a loading control either 48 hr or 96 hr after infection with Flag-Erk1 virus (**D**). These blots were all blotted and imaged together to facilitate direct comparison of expression levels at the two time points (**D**). Scale bar for (**A**) = 100 μ m. Scale bar for (**B**) = 50 μ m. [Please click here to view a larger version of this figure.](#)

Once the optimal virus dilution has been determined, purified OPCs were infected and cultured for a minimum of 48 h to allow transgene expression to reach sufficient levels, then seeded onto DRG neurons to assess their myelination potential (**Figure 5**). Once seeded onto DRG neurons, OPCs will differentiate into oligodendrocytes, and begin to express myelin proteins, which can be assessed by western blotting (**Figure 5C**). Some mature oligodendrocytes will myelinate, and this can be visualized through MBP staining (**Figure 5D**). Axons also need to be stained with a marker such as Neurofilament or β III-tubulin (**Figure 5D**) to ensure axon density is taken into account when analyzing myelination levels.

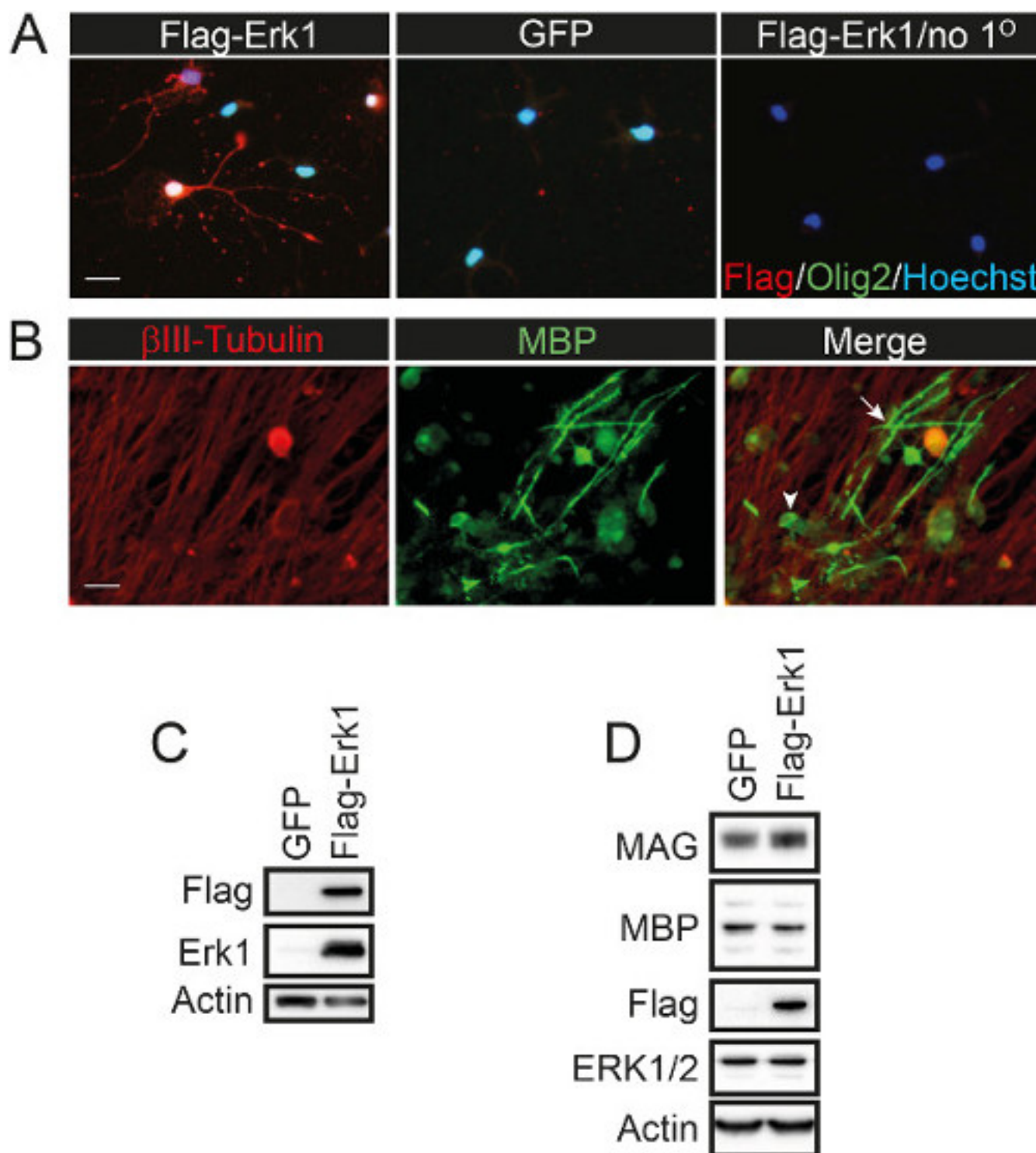


Figure 5: OPCs were infected with Flag-Erk1 virus for 48 hr before seeding onto DRG neurons. Sister OPCs were either fixed and stained for Flag and GFP (A) or lysed and probed for Flag and Erk1 to verify expression of the virally-overexpressed protein (C). Co-cultures were fixed after 2 weeks in culture and stained for β III-tubulin and MBP to visualize DRG axons and myelinating as well as non-myelinating oligodendrocytes (B). A myelinating oligodendrocyte is indicated by the arrow and a non-myelinating oligodendrocyte is indicated by the arrow-head (B). Parallel co-cultures were lysed and probed for the myelin proteins MBP and MAG, as well as Flag and Erk1/2 to confirm transgene expression in the co-culture (D). An increase in Erk1 levels was not visible in the co-culture due to the large quantities of DRG-derived Erk1 present in the lysates Scale bar = 20 μ m.

Name	Ingredients	Notes
TE Buffer pH 8	10 mM Tris pH 8 1 mM EDTA pH 8	Make up in deionized water.

Polyethylenimine (PEI)	1 g/L	Make up in deionized water, filter-sterilize and store stocks at -20 °C.
LB Medium	20 g Tryptone 10 g Yeast extract 10 g NaCl	Make up to 2 L with deionized water
50% Glycerol stock	Glycerol	Make up with an equal volume of deionized water and autoclave
HEK 293 T cell medium	DMEM 10% Fetal Bovine Serum 1% Penicillin/streptomycin 1% L-glutamine	
TNE lysis buffer	10 mM Tris pH 8 150 mM NaCl 1 mM EDTA pH 8 1% NP40	Dissolve NP40 in a smaller volume of deionized water first as it will crystallize on contact with water. Make up to final volume in deionized water
M1	MEM 10% Fetal Bovine Serum 0.4% D-glucose 2 nM L-glutamate 1% Penicillin/streptomycin	For use with rat DRG neurons
mM1	Neurobasal medium 2% B27 (SM1) supplement 0.4% D-glucose 2 nM L-glutamate 1% Penicillin/streptomycin	For use with mouse DRG neurons
M2	DMEM 10 mg/L Transferrin 5 mg/L Insulin 20 nM Progesterone 100 µM Putrescine 10 µM FdU 10 µM Uridine	Make up M2 in a larger volume DMEM without FdU and uridine for use over 1-2 months. Add FdU and uridine to smaller volumes that can be finished within 2 weeks
mM2	mM1 10 µM FdU 10 µM Uridine	Add FdU and uridine to smaller volumes of medium that can be finished within 2 weeks.
10x MT-PBS pH 7.4	28.48 g/L (160 mM) Na ₂ HPO ₄ ·2H ₂ O 5.52 g/L (40 mM) NaH ₂ PO ₄ ·H ₂ O 87.66 g/L (1.5 M) NaCl	Add to deionized water and adjust pH to 7.4 with 10 N NaOH
1x MT-PBS	10x MT-PBS Deionized water	Dilute 10x MT-PBS 1:10 in deionized water to make 1x MT-PBS
10x Borate buffer (1.5 M)	18.55 g Boric acid 1x MT-PBS	Dissolve boric acid in 150 ml 1x MT-PBS. Adjust to pH 8.56 with 10 N NaOH and bring final volume to 200 ml with 1x MT-PBS. Autoclave
1x Borate buffer (0.15 M)	10x Borate buffer 1x MT-PBS	Prepare 100 ml of this buffer to dissolve PLN in. Add 10 ml of 10x borate buffer (pH 8.56) to 90 ml of 1x MT-PBS
0.5 mg/ml Poly-L-ornithine (PLN)	50 mg Poly-L-ornithine Hydrobromide 0.15 M Borate buffer (pH 8.56)	Dissolve 50 mg of poly-L-ornithine hydrobromide in 100 ml of 1x borate buffer. Filter sterilize (0.22 µm filter) and store at 4 °C for up to one month
100x Poly-D-lysine (PDL)	5 mg Poly-D-lysine Sterile, deionized water	100x PDL stocks can be frozen at -20 °C in single-use aliquots. Upon use, dilute to 1x in sterile, deionized water
Papain buffer	See Table 2	
DNase	12,500 U DNase I 1 ml EBSS	On ice, dissolve the Dnase I in 1 ml of chilled EBSS. Aliquot (e.g., 300 µl/tube) and freeze overnight at -20 °C. Store at -20 °C.
4% BSA	8 g BSA 200 ml DPBS	Dissolve the BSA in 150 ml DPBS at 37 °C. Adjust the pH to 7.4 with ~1 ml of 1 N NaOH.

		Bring the volume to 200 ml. Filter through a 0.22 μ m filter to sterilize. Make 1 ml aliquots and store at -20 °C.
10x Lo Ovomucoid	3 g BSA 200 ml DPBS 3 g Trypsin inhibitor	Add BSA to 150 ml DPBS and mix well. Add trypsin inhibitor and mix to dissolve. Add ~1 ml of 1 N NaOH to adjust the pH to 7.4. Bring the volume to 200 ml with DPBS. Filter-sterilize through a 0.22 μ m filter. Make 1 ml aliquots and store at -20 °C.
6x Hi Ovomucoid	6 g BSA 200 ml DPBS 6 g trypsin inhibitor	Add BSA to 150 ml DPBS and mix well. Add trypsin inhibitor and mix to dissolve. Add ~1 ml of 1N NaOH to adjust the pH to 7.4. Bring the volume to 200 ml with DPBS. Filter-sterilize through a 0.22 μ m filter. Make 1 m aliquots and store at -20 °C.
SATO base	See Table 3	
SATO media (rat)	1% SATO base 1% Penicillin/streptomycin 1% 0.5 mg/ml Insulin 1% L-Glutamine 0.1% NAC 0.1% Biotin Make up with DMEM and filter sterilize.	
SATO media (mouse)	1% SATO base 1% 0.5 mg/ml insulin 1% Penicillin/streptomycin 1% L-Glutamine 0.1% NAC 0.1% Biotin 0.1% Trace Elements B 2% B27 Make up with DMEM and filter sterilize.	
Insulin	10 mg Insulin 20 ml Sterile deionized water	Add insulin to deionized water and add 100 μ l of 1 N HCl to allow the insulin to dissolve. Mix well. Filter through a 0.22 μ m filter and store at 4 °C for 4-6 weeks
NAC (N-Acetyl-L-cysteine)	5 mg/ml NAC DMEM	Dissolve NAC in DMEM to make a 5 mg/ml solution, aliquot and store at -20 °C.
d-Biotin	50 μ g/ml biotin Sterile, deionized water	Dissolve biotin in water to make a 50 μ g/ml solution, aliquot and store at -20 °C.
CNTF (ciliary neurotrophic factor)	10 μ g/ml CNTF 0.2% BSA in DPBS	Dilute CNTF to make a 10 μ g/ml solution with sterile 0.2% BSA in DPBS. Aliquot, flash freeze in liquid nitrogen and store at -80 °C.
PDGF (platelet derived growth factor)	10 μ g/ml PDGF 0.2% BSA in DPBS	Dilute PDGF master stock (prepared according to manufacturer's instructions) to 10 μ g/ml with sterile 0.2% BSA in DPBS. Aliquot, flash freeze in liquid nitrogen and store at -80 °C.
NT3 (Neurotrophin-3)	1 μ g/ml NT-3 0.2% BSA in DPBS	Dilute NT3 master stock (prepared according to manufacturer's instructions) to 1 μ g/ml with sterile 0.2% BSA in DPBS. Aliquot, flash freeze in liquid nitrogen and store at -80 °C.
Forskolin	50 mg Forskolin Sterile DMSO	Add 1 ml sterile DMSO to the 50 mg bottle of forskolin and mix well to resuspend fully. Transfer to 15 ml tube and add 11 ml sterile DMSO to reach a concentration of 4.2 mg/ml. Aliquot and store at -20 °C.

Table 3. Stock solutions.

Discussion

Myelination of axons is a crucial process for the optimal function of both the central and peripheral nervous systems of vertebrates. The generation and maintenance of myelinated axons is a complex and coordinated process involving molecular interactions between neuronal, glial

(from Schwann cells or oligodendrocytes) and extra cellular matrix proteins. The significance and applicability of this protocol is that it allows manipulation of proteins in one specific cell type within the mixed co-culture settings. As multiple cell types are involved in myelination both *in vivo* and in the *in vitro* myelination assay, using blunt pharmacological tools to block the activity of particular proteins or signaling pathways cannot provide specific information about the influence that proteins exert upon myelination from either a neuronal or glial perspective, unless the protein is uniquely expressed by one cell type. Thus, the *in vitro* myelination assay in conjunction with the use of Lentivirus to specifically transduce OPCs allows us to interrogate the effects that oligodendroglial proteins exert upon myelination. We have successfully used 2K7 Lentivirus to overexpress particular proteins specifically in oligodendrocytes for the *in vitro* myelination assay in order to dissect the effect that oligodendroglial signals exert upon CNS myelination⁹.

To achieve reproducible results in the transduced OPC *in vitro* myelination assay, optimization is required for each step of the protocol from cloning through dissections and OPC seeding. It is imperative that, at the level of DNA, the pENTR vector containing the tagged gene of interest construct is sequenced and that the 2K7 DNA with the tagged gene of interest is checked by western blot for expression of the tag and the gene of interest, prior to using the DNA to generate virus. It is essential to choose either HEK293T cells or HEK293 FT cells to generate the virus and grow under mycoplasma free environment. After producing lentivirus it is important to titrate each batch of virus to determine the optimum dilution to use on OPCs. This can be done by adding a range of viral dilutions to HEK293T cells or to OPCs. As the 2K7 construct contains both the gene-of-interest and GFP, efficacy of transduction can be assessed by expression of GFP by fluorescence microscopy and by assaying expression of the tag and the gene of interest by western blot analysis. Brighter GFP+ cells will become visible with higher virus concentrations, as it is possible for multiple viral particles to infect a single cell. Verification of the expression of GFP via fluorescence microscopy is a helpful indicator of titer of virus and expression of GFP but cannot be used as a surrogate for checking the expression of the protein of interest. It is therefore critical to verify the expression level of the protein of interest in both HEK293T cells and OPCs by western blot analysis. If the protein of interest is expressed endogenously by neurons in the co-cultures, the overexpression by OPCs/OLs may be masked when analyzing the co-culture lysate by western blot; thus it is important to either tag the protein of interest or culture the sister OPCs following seeding so expression of the protein of interest can be verified in the sister OPCs if not in the co-culture.

The quality of both DRG and OPC culture directly determines if the experiments succeeds. In the coculture settings, rat DRG neurons can be cocultured with OPCs derived from either rat or mouse. OPCs require gentle and swift handling. It is important to optimize the virus titer for OPCs as too little fails to effectively transduce the OPCs leading to low levels of expression of the protein of interest and too much virus is toxic to OPCs so there are too few cells to seed onto the neurons. Both of these sub optimal transductions may result in unanalyzable or insignificant changes in myelination or myelin protein expression. OPCs differentiate if they become over confluent and cannot be seeded after they have differentiated, thus the number of OPCs cultured and transduced needs to be optimized to local conditions. It is desirable to seed the same number of OPCs for each myelination assay allowing direct comparisons of the number of myelinated axonal segments to be compared between conditions over multiple experiments.

A potential limitation of this technique is the variability in basal myelination levels between myelination assays. Following viral transduction, the basal level of myelination is reduced, suggesting that virally infected OPCs do not myelinate as well as the naive (non-viral infected) OPCs. This can be overcome by quantifying the extent of myelination relative to the basal myelination in each assay. Thus, the empty vector that expresses only GFP must be used as an internal control. In addition to myelination, the expression of a protein of interest may also influence other aspects of OPCs such as survival, proliferation and differentiation, leading to an indirect effect on myelination. Therefore, analysis of OPC behavior such as cell viability should be undertaken in parallel to myelination assays.

The viral transduction methods described here are not limited to the study of oligodendrocyte myelination; in fact it can be generalized and applied to study other cell types such as Schwann cells, astrocytes, and neurons, while optimization may be required for individual cell type. This protocol offers great flexibility to study cell behavior such as proliferation and differentiation by selective overexpression of a protein of interest (wild type or mutant) in the desired cell type, which has particular advantages when using a mixed cell culture system. The cells that are used for transduction can be isolated from either rat or mouse (wild type or transgenic), thus allowing for the targeted study of signaling and compensation mechanisms in transgenic animals, which is usually difficult to achieve and much more time consuming than using an *in vivo* transgenic mouse model. Recent evidence suggest that the lentiviral-based strategy has been successfully used for cell transduction in animal models¹³, suggesting a strong potential of transducing oligodendroglial cells using the lentiviral approaches *in vivo*.

In conclusion, combining the *in vitro* myelination assay with Lentiviral infection of OPCs provides a strategic tool for the analysis of molecular mechanisms involved in myelination. The role of specific proteins in myelination can be interrogated and as OPCs can be isolated from rats and mice for use on rat DRG co-cultures, the lentiviral approach can also be used in combination with knockout technology of various mouse lines to interrogate mechanisms of compensation in the processes of myelination.

Disclosures

The authors declare that there is no conflict of interest regarding this research.

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