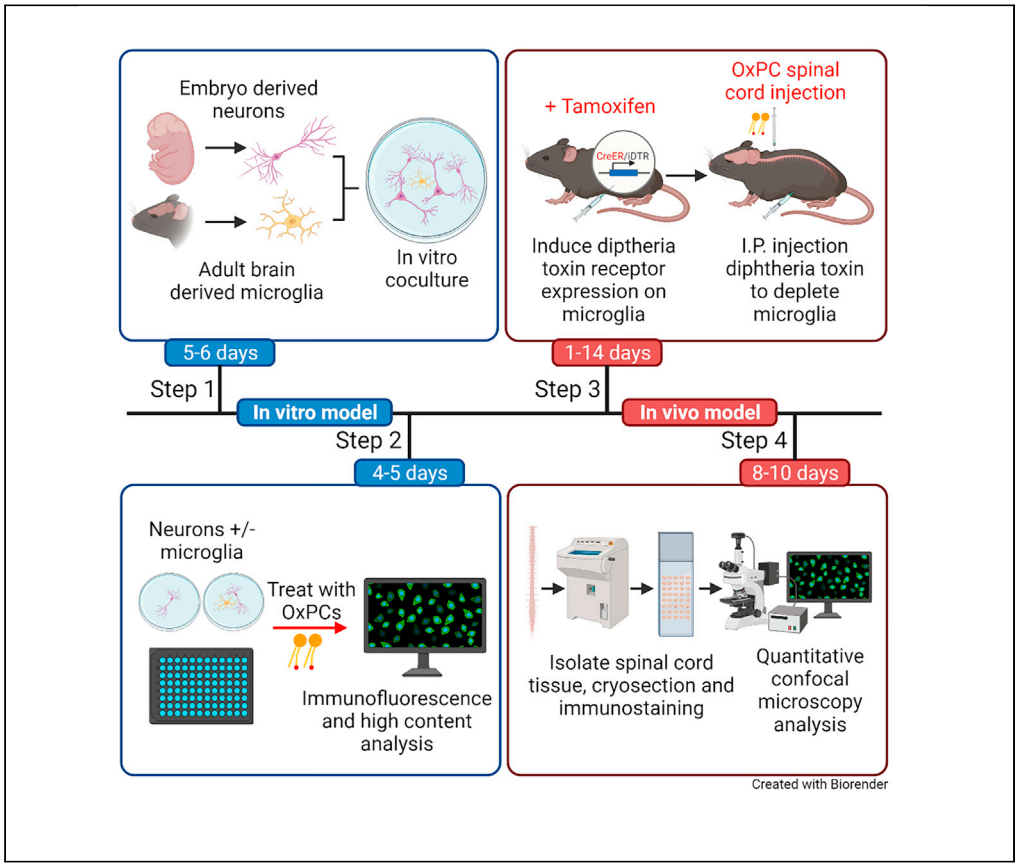
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Studying the microglia response to oxidized phosphatidylcholine in primary mouse neuron culture and mouse spinal cord



Oxidized phosphatidylcholine (OxPC) found in multiple sclerosis brain lesions mediates neurodegeneration. Microglia are prominent responders to the OxPC insult, and thus, studying their protective or noxious functions is important to help halt neurodegeneration. Here, we present protocols including cell isolation and culture, animal surgeries, as well as tissue processing and isolation to study the microglia response to OxPC-mediated neurodegeneration *in vitro* and *in vivo*.

Yifei Dong, Brian M. Lozinski, Claudia Silva, V. Wee Yong

vyong@ucalgary.ca

Highlights

Co-culture of primary mouse neurons and microglia for high-throughput assays

Model neurodegeneration in the spinal cord with oxidized phosphatidylcholine injection

In vitro and *in vivo* approaches to study roles of microglia during neurodegeneration

Dong et al., STAR Protocols 2, 100853
December 17, 2021
https://doi.org/10.1016/j.xpro.2021.100853

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Studying the microglia response to oxidized phosphatidylcholine in primary mouse neuron culture and mouse spinal cord

Yifei Dong,[1,2] Brian M. Lozinski,[1] Claudia Silva,[1] and V. Wee Yong[1,3,*]

[1]Hotchkiss Brain Institute and the Department of Clinical Neuroscience, University of Calgary, Calgary, AB T2N 4N1, Canada

[2]Technical contact

[3]Lead contact

*Correspondence: vyong@ucalgary.ca
https://doi.org/10.1016/j.xpro.2021.100853

SUMMARY

Oxidized phosphatidylcholine (OxPC) found in multiple sclerosis brain lesions mediates neurodegeneration. Microglia are prominent responders to the OxPC insult, and thus, studying their protective or noxious functions is important to help halt neurodegeneration. Here, we present protocols including cell isolation and culture, animal surgeries, as well as tissue processing and isolation to study the microglia response to OxPC-mediated neurodegeneration *in vitro* and *in vivo*.

For complete details on the use and execution of this protocol, please refer to Dong et al. (2021).

BEFORE YOU BEGIN

All experiments were conducted with ethics approval from the Animal Care Committee at the University of Calgary under regulations of the Canadian Council of Animal Care.

Prepare CX3CR1^{creER}:Rosa26^{iDTR} mice

Timing: at least 10–12 weeks prior to experiment

1. Cross CX3CR1$^{creER/creER}$ mice (JAX strain 021160) with Rosa26$^{iDTR/iDTR}$ mice (JAX strain 007900) to obtain the CX3CR1^{CreER}:Rosa26^{iDTR} mice.

Prepare tamoxifen solution

Timing: 2 h

2. Place tamoxifen (T5648, Sigma) in corn oil by shaking at 55°C for 1–2 h until it dissolves completely, at 20 mg/mL.

⚠ CRITICAL: Tamoxifen is known to be a human carcinogen and should be handled with care.

Induce diphtheria toxin receptor (DTR) expression in microglia

Timing: 5 days

3. At least 3 weeks prior to start of experiment, inject 100 μL of tamoxifen (20 mg/mL) intraperitoneally once daily in CX3CR1^{CreER}:Rosa26^{iDTR} mice for 5 consecutive days.

⚠ CRITICAL: Tamoxifen is injected 3 weeks prior to start of experiment to ensure that DTR at the time of experiment is only expressed on long living microglia in the central nervous system and not on peripheral monocytes, which are regularly replaced by progenitors from the bone marrow.

Prepare diphtheria toxin solution

Timing: 10 min

4. Dissolve lyophilized diphtheria toxin from *Corynebacterium diphtheriae* (D0564-1MG, Sigma) in sterile distilled water (1 mg/mL). Solution can be stably stored at −20°C in the dark.

⚠ CRITICAL: Diphtheria toxin is highly toxic and should be handled with extreme care.

Prepare the OxPC solutions

Timing: 10 min

5. Prepare OxPC solutions with 1-O-palmitoyl-2-O-(5-oxovaleryl)-sn-glycero-3-phosphocholine (POVPC Avanti Polar Lipids, 870606P) or 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PAzePC Avanti Polar Lipids, 870600P), by dissolving 1 mg of lyophilized powder in 100 μL of sterile solvent to a final concentration of 10 mg/mL.
   a. For cell culture experiments, dissolve purified OxPCs in 100% ethanol prior to use. The solution can be stored at −20°C and should be used within 2 weeks.
   b. For *in vivo* spinal cord injection, dissolve purified OxPCs in phosphate buffer saline (PBS) prior to use. The solution can be stored at −20°C and should be used within 3 days.

Note: OxPCs are highly unstable and should be stored as lyophilized powder until just before the start of the experiment. For *in vitro* cell culture experiments, non-oxidized dipalmitoylphosphatidylcholine (DPPC, Sigma) dissolved in 100% ethanol should be used as the negative control. However, DPPC does not dissolve in aqueous buffers such as PBS, and thus PBS injected mice are used as sham controls for *in vivo* spinal cord experiments.

Prepare 4% paraformaldehyde (PFA) solution

Timing: 2–3 h

6. Prepare 16 % PFA solution by dissolving PFA powder in PBS.
   a. In a glass beaker or wide mouthed glass bottle, add 1 small pellet of solid NaOH (Sigma) and a magnetic stir bar to 70 mL of ddH_2O.

Note: NaOH pellet is added to aid the dissolving of PFA powder.

   b. Place the beaker/bottle on a magnetic stirrer hot plate inside a fume hood. Turn on stirring and low heat.
   c. While stirring, slowly add 16 g of PFA powder to the solution.

Note: PFA is a fixative and thus should be handled with care.

   d. Continue stirring and heat the solution to ~60°C until the PFA completely dissolves.

e. Add 5 mL of 20X PBS and cool to 22°C–24°C.
f. Measure pH and adjust to between 7.0–7.6.
g. Prior to use, dilute 1:4 with 1X PBS, otherwise store at 4°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse E06 anti-OxPC (5 μg/mL)	Avanti/Sigma	Cat# 330001S-100ug
Rabbit anti-human/mouse IBA1 (1:1000 from stock)	FUJIFILM Wako	Cat# 019-19741
Mouse anti-human/mouse tubulin β3 (1:500 from stock)	BioLegend	Cat# 801202
Rat anti-mouse CD68 (1:500 from stock)	BioLegend	Cat# 137002
Rabbit anti-mouse TMEM119 (1:200 from stock)	Abcam	Cat# Ab209064
Goat anti-human/mouse Olig2 (1:200 from stock)	R&D Systems	Cat# AF2418
Rabbit anti-mouse NFH (1:1000 from stock)	EnCor Biotechnology	Cat# RPCA-NF-H
Rat anti-mouse CD16/32 (1:200 from stock)	BD Pharmingen	Cat# 553142
Alexa Fluor 488 donkey anti-mouse IgM (1:400 from stock)	Jackson ImmunoResearch	Cat# 715-545-140
Cyanine Cy3 donkey anti-rat IgG (1:400 from stock)	Jackson ImmunoResearch	Cat# 712-165-153
Alexa Fluor 488 donkey anti-goat IgG (1:400 from stock)	Jackson ImmunoResearch	Cat# 705-545-003
Alexa Fluor 647 donkey anti-rabbit IgG (1:400 from stock)	Jackson ImmunoResearch	Cat# 711-605-152
Chemicals, peptides, and recombinant proteins		
PAzePC	Sigma/Avanti	870600P
POVPC	Sigma/Avanti	870606P
Diphtheria toxin	Sigma	D0564
Tamoxifen	Sigma	T5648
Percoll	Sigma	P1644
Paraformaldehyde powder	Sigma	158127
Fluoromount-G solution	Thermo Fisher	00-4958-02
FSC 22 Frozen Section Media	Leica	3801480
B-27™ Plus Neuronal Culture System	Thermo Fisher	A3653401
HBSS, no calcium, no magnesium, no phenol red	Thermo Fisher	14175095
Critical commercial assays		
EasySep™ Mouse CD11b Positive Selection Kit II	STEMCELL Technologies	18970
Experimental models: Organisms/strains		
OxPC spinal cord injection, C57Bl/6 mice (6–10 week old, male or females)	Charles River	C57BL/6NCrl
Primary neuron (embryonic days 14-16) and microglia culture (6–10 week old, male or females), C57Bl/6 mice	Charles River	C57BL/6NCrl
Microglia depletion, CX3CR1^{CreER}:Rosa26^{iDTR} mice (6–10 week old, male or females)	JAX, bred in house	Cross Stock #: 021160 with 007900
Software and algorithms		
Fiji ImageJ	NIH	https://imagej.net/Fiji
MetaXpress High-Content Image Acquisition and Analysis Software	Molecular Devices	https://www.moleculardevices.com/products/cellular-imaging-systems/acquisition-and-analysis-software/metaxpress
Leica Application Suite X	Leica	https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/
cellSens	Olympus	https://www.olympus-lifescience.com/en/software/cellsens/powerful-analysis-tools/

STEP-BY-STEP METHOD DETAILS

Isolate and culture primary mouse cortical neurons

Timing: 4–6 days

Primary neurons are isolated from brains of embryonic C57BL/6 mice and grown in preparation for co-culturing with microglia. Here, we propose to isolate and grow neurons from embryos as it is difficult to isolate viable neurons from adult mice for large scale experiments. On average each pregnant C57BL/6 mouse carries 6 embryos, if additional cells are needed; CD-1 mice may be considered as they have a higher average litter size of 10.

1. Coat T25 tissue culture flasks with 5 mL of 10 μg/mL poly-L-ornithine (PO) dissolved in sterile distilled water for at least 30 min (preferably more than 8 h) at 22°C–24°C. Remove the PO solution and wash with sterile distilled water.
   a. For downstream assay, coat 96-well flat bottom black/clear plates (Falcon 353219) with PO solution. This may also be used for immediate culturing of neurons.

2. Prepare the following:
   a. Prepare and prewarm the growth medium containing Neurobasal Plus medium (Gibco) and 1X B27 Plus Supplement (Gibco) at 37°C.

   Note: Prepare this fresh before use.

   b. On a hot plate, prewarm ~ 300 mL of water in a 1 L beaker to 35°C–38°C.
   c. Disinfect instruments with 70% ethanol: dissection scissors, forceps, iridectomy scissors, ultrafine point forceps, and scalpel.
   d. Autoclaved 100 mL bottles and Buchner funnel with nylon mesh at 130 μm pore size.

3. Acquire cortical neurons from mouse embryos at gestation days 14–16.
   a. Euthanize pregnant mouse with ketamine (100 mg/mg)/xylazine (10 mg/kg) overdose via intraperitoneal injection.
   b. Lay the anesthetized mouse on its back; disinfect the abdominal area with 70% ethanol. Lift the skin of the lower abdomen and make a V-shaped incision with scissors.
   c. Disinfect the instruments with 70% ethanol once more. Then lift the uterine horns containing the embryos, dissect them out of the abdominal cavity, and place them in a sterile 10-cm petri dish containing calcium and magnesium-free Hank's Balanced Salt Solution (CMF-HBSS).
   d. In a biosafety cabinet, dissect the uterine horns to release the embryos from their individual sacs.
   e. Use iridectomy scissors to cut around the base of the head of each embryo, such that the skin can be lifted to reveal the brain. Carefully scoop out the brain into a new sterile 10-cm petri dish containing 20 mL CMF-HBSS.
   f. Remove the meninges from the brains using a dissection microscope, separate the cerebral hemispheres and place them in a new sterile 10-cm petri dish containing 3–5 mL of CMF-HBSS.
   g. Dice the cerebral hemispheres with a sterile scalpel into fragments of < 1 mm and form a tissue suspension by adding 10–15 mL of CMF-HBSS. Drop a stir-bar into an autoclaved 100 mL bottle, then transfer the tissue suspension into the bottle with a sterile pipette. Note the volume transferred.
   h. Add sterile trypsin to the tissue suspension to a final concentration of 0.025% (from 2.5% stock solution, Gibco). Immerse the 100 mL bottle containing the tissue suspension in the prewarmed water bath at 35°C–38°C, set the stirring speed to low medium, and homogenize the tissue for 15 min at 35°C–38°C.
   i. Add 1 mL of sterile heat inactivated FBS to the homogenized suspension to inactivate the trypsin, then pour the suspension through the Buchner filter funnel with nylon mesh into a new autoclaved 100 mL bottle. Using the plunger of a 3 mL syringe, gently grind the tissue on the nylon mesh and wash it through the filter with CMF-HBSS.

   Note: Alternatively, a sterile 100 μm cell strainer may be used to filter cells.

j. Transfer the filtered tissue suspension to 50 mL tubes and centrifuge at 300 × *g* for 5 min at 22°C–24°C.
k. Discard the supernatant and resuspend the cell pellets in CMF-HBSS, pooling all the cell suspensions in one 50 mL tube, then top up with CMF-HBSS to 50mL, and centrifuge at 300 × *g* for 5 min at 22°C–24°C.
l. Discard the supernatant and resuspend the cell pellet in 5–10 mL of prewarmed growth medium, then count cells with trypan blue for culturing.

⚠ CRITICAL: Gently handle neuronal cells after dissociation from the brain as these cells are highly sensitive to stress and damage. Do not vortex or vigorously triturate.

4. Culture neurons in preparation for microglia co-culture.
   a. Seed 20 × 10^6 cells isolated from step 3 per PO-coated T25 flask from step 1 in 5 mL of growth medium. Incubate cells at 37°C in a humidified atmosphere of 95% air and 5% CO_2.
   b. After 2–3 days, healthy neurons have grown processes and can be harvested for experimental use.
      i. Add trypsin to the neuron culture to a final concentration of 0.25%. Incubate at 37°C for ~ 5 min to dislodge the neurons, then add 0.5 mL of sterile heat inactivated FBS.
      ii. Transfer the cell suspension to a 15 mL tube, centrifuge at 300 × *g* for 5 min at 22°C–24°C. Discard the supernatant and resuspend in 5 mL of prewarmed growth medium.
      iii. Count cells with trypan blue and seed the neurons into PO coated 96-well flat bottom black/clear plates from step 1 at 0.75–1 × 10^5 cells per well in 100 µL of growth medium.
   c. After 24–48 h, Neurons with healthy process formation in 96-well plates can be co-cultured with microglia (troubleshooting 1).
   d. Alternatively, if under time constraint, neurons isolated in step 3 may be directly seeded onto PO coated 96-well plates for use.

Note: This will result in greater amounts of debris in the neuron culture.

Isolate microglia from the adult mouse brain

Timing: 5 h

Primary microglia isolated from the brains of 6–12 weeks old C57BL/6 mice will be cocultured with embryonic neurons. We propose the use of adult brain derived microglia to better model *in vivo* microglia; alternatively, microglia may be enriched from mixed glia cultures generated from the brains of neonatal mice. These steps are in accordance with the manufacturer's protocols for the EasySep™ Mouse CD11b Positive Selection Kit II from STEMCELL Technologies (https://cdn.stemcell.com/media/files/pis/10000003693-PIS_01.pdf).

Note: We have also used this protocol to isolate microglia from the brains of 52-week-old mice.

5. Prepare the following:
   a. Sample preparation medium of CMF-HBSS containing 2% FBS and 1 mM EDTA.
   b. Prewarm brain digestion medium containing CMF-HBSS with 20 units/mL of Papain (Worthington) and 100 µg/mL DNase I to 22°C–24°C.

Note: Prepare 3 mL brain digestion medium for up to 3 brains. For 4 or more brains, prepare 1 mL brain digestion medium per brain.

c. 100% isotonic Percoll solution by combining 9 parts of Percoll (Sigma) with 1-part of 10X D-PBS (37354, STEMCELL Technologies).
d. Combine 3 parts of 100% isotonic Percoll with 7 of parts sample preparation medium to make 6 mL of 30% Percoll solution per brain.
e. Disinfect dissection tools with 70% ethanol: dissection scissors, forceps, iridectomy scissors, small dissecting spatula, scalpel.

6. Harvest brains from mice.
   a. Euthanize mice with ketamine (100 mg/kg) /xylazine (10 mg/kg) overdose via intraperitoneal injection.
   b. In a biosafety cabinet, disinfect mouse with 70% ethanol, then decapitate it using dissection scissors.
   c. Use iridectomy scissors to cut alone the top of the skull, and in parallel on both sides.
   d. Use forceps to peel away the skin and top the skull, then use the dissecting spatula to scoop out the brain.
   e. Place the brain in a sterile petri dish containing 1 mL of brain digestion medium.

7. Isolate single cell suspension from brains. All centrifugation steps can be performed at 22°C–24°C or 4°C.
   a. In a biosafety cabinet, mince the brains with a scalpel into small pieces < 1 mm.
   b. Transfer the minced brain tissue into a 50 mL tube, then rinse the petri dish with the remaining brain digestion medium and add this to the cell suspension in the 50 mL tube.
   c. Incubate the cell suspension at 37°C for 30 min while continuous shaking or rotating at medium low speed so the minced brain tissues remain dispersed in the digestion medium.
   d. Filter the digested brain tissue through a 70 μm nylon mesh strainer using the rubber end of a syringe plunger, then rinse the strainer with sample preparation medium.

   Note: New strainers may be used if necessary.

   e. Centrifuge at 300 × *g* for 10 min with low brakes. Then carefully remove and discard the supernatant with a serological pipette.
   f. Add 30% Percoll prepared in step 5d to the cell pellet.

   Note: Use one or multiple 15 mL tubes for volume < 30 mL.

   g. Centrifuge at 700 × *g* for 10 min with no brakes.
   h. Use a 2 mL serological pipette and carefully remove the upper myelin layer, then carefully discard the remaining supernatant.
   i. Transfer cells to a new tube and top up with sample preparation medium. Centrifuge at 300 × *g* for 10 min with low brakes.
   j. Discard the supernatants, then count with trypan blue and resuspend cells at 2.5 × 10^7 cells/mL in PBS containing 2% FBS and 1 mM EDTA.

8. Enrich for $CD11b^+$ microglia from cells isolated from brains using the EasySep™ Mouse CD11b Positive Selection Kit II (troubleshooting 2).
   a. Perform steps in accordance with the manufacturer's protocol, using the EasySep™ magnet.
   b. Resuspend enriched cells in neuron growth medium and count with trypan blue.

   Note: Expect 1–2 × 10^6 cells from 3 brains.

9. Add microglia to neurons cultured in 96-well plates from step 4.
   a. 2.5 × 10^4 microglia resuspended in 100 μL of neuron growth medium are added to each well containing 0.75–1 × 10^5 neurons.

b. Neurons with microglia are now cocultured in a total volume of 200 μL of neuron growth medium.
c. Cocultured microglia and neurons are used for experiments after 24 or 48 h.

Note: Prepare 3–4 technical replicate wells per experimental condition as required.

Assess microglia function and OxPC-mediated neurotoxicity in the microglia neuron coculture

Timing: 3 days

Cocultured microglia and neurons are treated with OxPC and/or other modulators of microglia to assess the effect of microglia on neuron survival in the context of OxPC mediated neurodegeneration. In addition, this coculturing method may also be adapted to test the neuroprotection or neurotoxic potential of other molecules.

10. Ensure microglia and neurons have been in coculture for at least 24 h.
    a. Include neurons alone, and untreated microglia and neurons as control conditions.
11. Add purified OxPCs (i.e., POVPC or PAzePC or other toxins) to the cells at desired concentrations (troubleshooting 3).
    a. Neurons and microglia are cocultured in 200 μL of neuron growth medium, in 96-well plates.
    b. Carefully remove 100 μL of the media while ensuring the tip of the pipette is to the edge of the well to minimize accidental removal of cells Then, add 100 μL of neural basal plus medium alone (no B27 plus supplement) containing 2X the concentration of OxPCs for a final 1:2 dilution.
       i. For example, for a final concentration of 50 μM of POVPC, add 100 μL of neural basal plus media containing 100 μM of POVPC to 100 μL of cells.
    c. Include the appropriate negative controls:
       i. Add equivalent volume of ethanol used to dissolve the OxPCs to neurons alone and neurons cocultured with microglia.
       ii. Add equivalent concentrations of DPPC as OxPCs to neurons alone and neuron cocultured with microglia.
12. Incubate cells at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Optional: Adding cell viability dyes such as Calcein AM (Thermo Fisher) or propidium iodide (Thermo Fisher) at manufacturer's recommended concentrations to OxPC treated neurons allows live tracking and imaging of neurotoxicity over time.

Optional: Treat cells with or without modulators of interest as needed. For example, to test whether microglia phagocytosis is needed to protect neurons for OxPCs, first remove 100 μL of neuron growth medium (from a total of 200 μL) from cells. Then add 50 μL of neural basal medium containing 20 μM (4X concentration) of cytochalasin D (Sigma) or 800 nM (4X concentration) of latrunculin A (Thermo Fisher) to cells and incubate at 37°C for 30 min. Finally, add 50 μL of neural basal plus medium alone (no B27 plus supplement) containing 4X the concentration of OxPCs for a final 1:4 dilution.

13. After 24 h, remove tissue culture supernatant and fix cells for immunochemistry and quantitative fluorescence microscopy.
    a. Tissue culture supernatant may be collected and stored at −80°C for additional secretome analysis.
    b. To fix cells, after the removal of the supernatant, add 100 μL of 4% PFA to cells and incubate for 10 min at 22°C–24°C.
    c. Carefully pipette to remove the PFA and wash twice with 100 μL of PBS.

Note: Cells may be incubated with microglia and treatments for longer periods of time (i.e. 48 h) if needed.

Pause point: The fixed cells in 96-well plates may be sealed with parafilm and stored at 4°C for at least 2 weeks prior to staining and analysis.

14. Permeabilize cells with 100 μL PBS containing 0.2% Triton X-100 (Sigma) for 10 min at 22°C–24°C.
15. Carefully pipette to remove the supernatant and block cells by adding 100 μL of Odyssey Blocking Buffer (LI-COR) for 1 h at 22°C–24°C.
16. Remove the blocking buffer and incubate with 70 μL of primary antibodies diluted in the blocking buffer for 8–12 h at 4°C with gentle shaking.
    a. Use mouse anti-tubulin β3 IgG (clone TUJ1, Biolegend) at 2 μg/mL to label neurons.
    b. Use rabbit anti-IBA1 IgG (polyclonal, Wako Fujifilm) at 0.5–0.7 μg/mL to label microglia.
17. Remove the supernatant and wash 2X with 100 μL of PBS.
18. Incubate with 70 μL of secondary antibodies and DAPI (Sigma) diluted in blocking buffer for 0.5–1 h at 22°C–24°C with gentle shaking.
    a. Use Alexa Fluor 488 donkey anti-mouse IgG and Alexa Fluor 647 donkey anti-rabbit IgG (Jackson ImmunoResearch) at 1:400 from manufacturer's recommended stock concentration.
    b. Use DAPI at 1 μg/mL.
19. Remove the supernatant, wash 2X with 100 μL of PBS, and resuspend cells in 100 μL of PBS.

Note: Neurons and microglia may be labeled with other primary and secondary antibodies of interest as needed for alternative analyses.

Pause point: The fixed and stained cells in 96-well plates may be sealed with parafilm and stored at 4°C for at least 2 weeks prior to analysis.

20. Image cells and quantify neuron survival in coculture with or without microglia after OxPC treatments.
    a. Use a 10×/0.5 NA air or a 20× 0.45 NA air objective on the ImageXpress Micro XLS High-Content Analysis System (Molecular Devices) with the appropriate detection filters to capture 9–12 imaging fields evenly distributed across each well per experimental condition to quantify neuron survival.
        i. Detection filters setup: DAPI excitation 387/11 – emission 447/60; Alexa Fluor 488 excitation 482/35 – emission 536/40; and Alexa Fluor 647 excitation 628/40 – emission 692/40.
        ii. Save all raw data files for analysis and record keeping.

Note: Cells may be imaged with any fluorescence widefield or confocal microscopes that support 96-well plates. Automated imaging platforms are recommended to conserve the experimentalist's time.

Induce OxPC-mediated neurodegeneration in the spinal cord white matter of microglia-sufficient and microglia-deficient mice

Timing: 1–15 days

Purified OxPCs are stereotaxically injected by surgery into the ventral spinal cord white matter of mice to induce demyelination and neurodegeneration. The needle injection site is in the ventral spinal cord white matter to minimize non-specific injury caused by the surgery, which may occur at varying severity in the dorsal white matter where the needle enters the spinal cord. The *in vivo* function of microglia responding to OxPCs may be assessed by injecting OxPCs into tamoxifen pre-treated

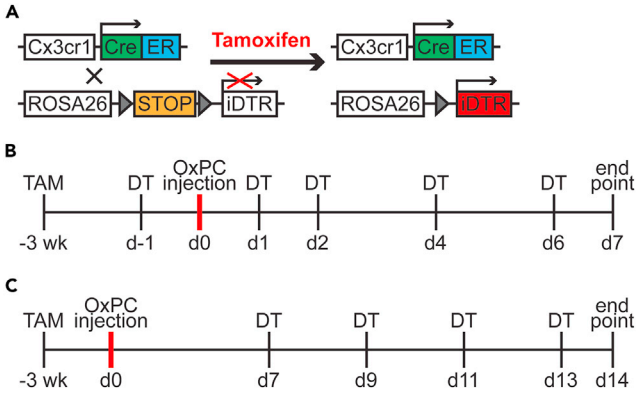


Figure 1. Schematic for DT induced microglia depletion following injection of OxPC into the spinal cord

(A) Tamoxifen treatment in CX3CR1^{CreER}:Rosa26^{iDTR} mice induces recombination and removal of stop codon to allow DTR expression on CX3CR1^{+} cells.

(B) Timeline of experiment for early DT induced microglia depletion following OxPC spinal cord injection.

(C) Timeline of experiment for late DT induced microglia depletion following OxPC spinal cord injection.

CX3CR1^{CreER}:Rosa26^{iDTR} mice with or without diphtheria toxin (DT) injection. While this method describes the injection of OxPCs, other neurotoxins, cytokines, or demyelinating agents can be injected using these steps.

21. Deplete microglia in tamoxifen pre-treated CX3CR1^{CreER}:Rosa26^{iDTR} mice (Figure 1A) by intraperitoneal injection of 100 μL of PBS (microglia sufficient controls) or PBS containing 1 μg of DT (microglia deficient treatments).
    a. To assess the effects of early microglia depletion (i.e., days 0–7), inject DT on the day prior to spinal cord surgery, the day of the surgery, and 1, 2, 4, and 6 days after the surgery (Figure 1B).
    b. To assess the effects of delayed microglia depletion (i.e., days 7–14), inject DT 7, 9, 11, and 13 days after the surgery (Figure 1C).

⚠ CRITICAL: Random littermates should be assigned to each experimental group to minimize potential microbiome effects. Experiments should contain at least 3 biological replicates per group, and repeated at least once for a total of 6 biological replicates over two experiments.

22. Prepare the surgery.
    a. Prepare the needle and the syringe.
        i. Place a 32-gauge Hamilton needle on a 10 μL Hamilton syringe.
        ii. Clean syringe in the following order with autoclaved 20 μL ddH_2O, PBS, and acetate.
        iii. Draw desired volume of PBS (sham controls) or OxPC (POVPC or PAzePC dissolved in PBS at 10 mg/mL) into syringe, be careful to avoid bubbles. At least 0.5 μL is needed per spinal cord injection per mouse.
        iv. Place syringe onto the stereotaxic instrument.
    b. Sterilize the following tools using microbead sterilizer at 120°C–130°C.
        i. #15 scalpel blade and scalpel
        ii. Micro dissecting spring scissors
        iii. Tissue spreader
    c. Anesthetize a mouse with 100 mg/kg ketamine and 10 mg/kg xylazine.
    d. Clean and prepare the surgical site.
        i. Use a shaver to remove hair from the ears to midback, revealing the surgical site.
        ii. Clean the site with 70% ethanol using a gauze pad.

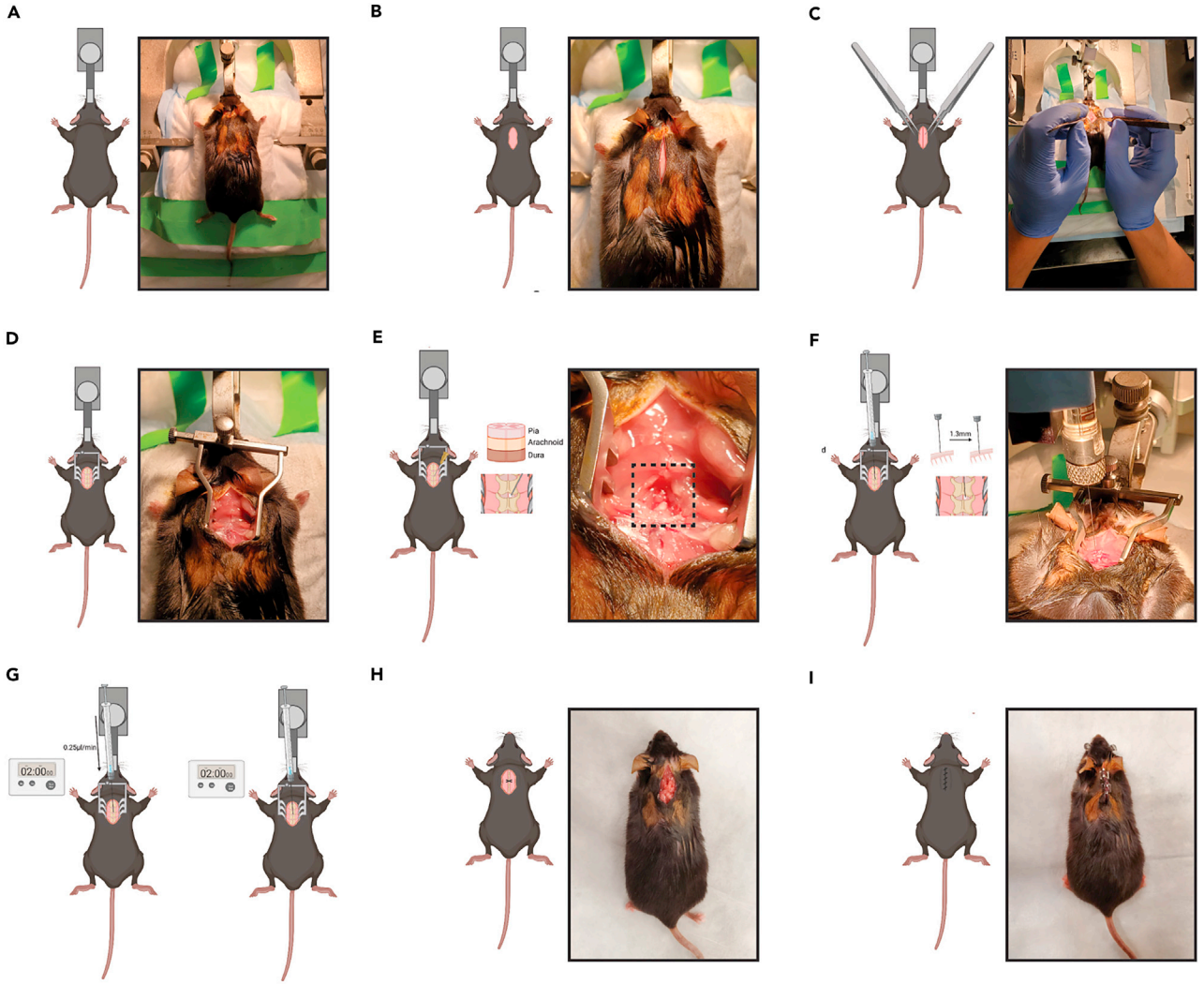


Figure 2. Schematic of the surgery to inject OxPCs into the ventral spinal cord white matter

(A) Mouse resting on surgery platform.
(B) Incision made on the back of mouse.
(C) Using forceps to pull apart the skin and fat.
(D) Using the spreader to keep the skin and fat separate.
(E) Exposing the spinal cord dorsal T3-T4 gap.
(F) Inserting the Hamilton needle into the spinal cord.
(G) Injection into the spinal cord.
(H) Suture together the muscle/fat pad.
(I) Using sutures or staples to close the incision on the back of the mouse.
Figure partially made with Biorender.

iii. Apply iodine to disinfect the surgical site.
iv. Apply ophthalmic gel to prevent eyes from drying out.

e. Once the mouse reaches a surgical plane of anesthesia, place it onto the stereotaxic instrument dorsal side up. To maintain the mouse's body temperature, use a heating pad underneath or heating lamp overhead. Elevate the midsection for easier access to the thoracic spinal region (Figure 2A).

23. Surgically expose the spinal cord at the T3-T4 vertebras.
    a. Make a 2 cm incision between the shoulder blades using a #15 scalpel (Figure 2B).

b. Pull apart the skin and fat pads underneath using two forceps (Figure 2C).
c. Use a spreader to firmly keep the skin and fat pads apart to expose the underlying musculature (Figure 2D).
d. Find the T2 vertebrae using forceps and countdown caudally to find the T3-T4 gap.
   i. T2 is an anatomical landmark in C57BL/6 due to its pronounced appearance.
e. Use the micro dissecting spring scissor and forceps to blunt dissect apart the musculature to expose the spinal cord at the T3-T4 gap (Figure 2E).
f. Use a 30-gauge needle to carefully prick and remove the meningeal layer.
   i. To ensure the ventro-lateral white matter is targeted use an overhead lamp to distinguish the dorsal white and gray matter. Remove the meninges at the boundary of the dorsal white matter.
g. Absorb potential blood or cerebral spinal fluid with eye spears to keep the operating area clear.

24. Inject OxPCs into the T3-T4 gap.
    a. Stereotactically angle the Hamilton syringe and needle to 0.5 degrees.
    b. Align the syringe over the T3-T4 gap with removed meninges.
    c. Lower the needle tip until it is contacting the spinal cord dorsal surface slightly to the right side of the midline (troubleshooting 4).
    d. Note the position of the needle using the graded measurements on the Z-direction stereotaxic arm as the baseline. From the baseline subtract 1.3 mm to reach the ventral white matter.
    e. Using the Z-direction arm control, make a quick downward motion to insert the needle into the spinal cord. (Figure 2F)
    f. Slow lower the needle until the baseline minus 1.3 mm position.
    g. Use the micromanipulator to inject 0.5 μL of PBS (sham control) or OxPC at a rate of 0.25 μL/min. This amounts to one rotation of the micromanipulator every 5 s. Leave the needle in place for 2 min to avoid backflow (Figure 2G).
    h. Carefully retract the needle from the spinal cord.
25. Post-Surgery recovery (troubleshooting 5).
    a. Make a single interrupted suture in the muscle/adipose tissue over the spinal column using vicryl suture (Figure 2H).
    b. Suture the skin using uninterrupted vicryl sutures or staples (Figure 2I).
    c. Provide any post-operative analgesic required by institutional animal care regulations.
    d. Place animals in heated recovery chamber until they regain conscious movement.
    e. Monitor animals for open sutures and as required by institutional animal care regulations.
26. Inject DT or PBS intraperitoneally following spinal cord surgery to compare neurodegeneration with or without microglia deficiency as needed (see step 21) (troubleshooting 6).
27. Mice may be euthanized to collect the spinal cord for analysis at any subsequent time points.

Collect spinal cords and generate fixed frozen tissue sections for analysis

Timing: 3–4 days

Dissection and isolation of spinal cords from mice for downstream analysis. The steps below describe how to collect and process spinal cords for histology and microscopy analysis. For cellular analysis of immune cells in the spinal cord lesion, we have also isolated the lesion containing spinal cord tissue (~3–4 mm region surrounding the injection site at the T3-T4 gap), and dissociated either enzymatically (i.e., with papain and DNase I) or mechanically (i.e., with a Dounce homogenizer) in appropriate buffer/medium, filtered sequentially with 70 μm and 40 μm cell strainers, myelin debris removed using the Debris Removal Solution (Miltenyi, according to manufacturer's instructions https://www.miltenyibiotec.com/CA-en/products/debris-removal-solution.html), and labeled with desired antibodies and/or viability dyes for downstream analysis such as flow cytometry or single cell RNA sequencing (not covered here).

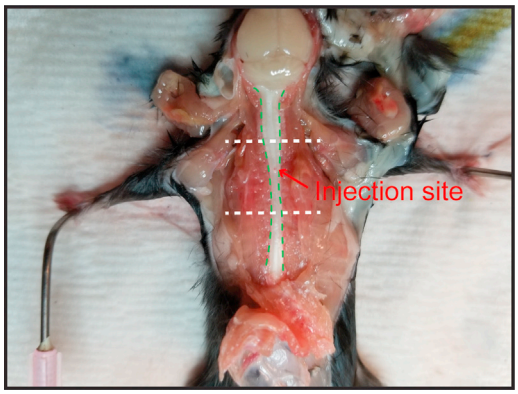


Figure 3. Exposed brain and spinal cord for tissue isolation

Dashed green lines indicate where to cut alone the spinal cord for tissue isolation. White lines indicate the section of the spinal cord collected for downstream processing and analysis. Red arrow points to a small red dot that is the injection site (this may not be clearly visible each time depending on the amount of meningeal injury caused by the initial surgery).

28. Prepare one 1.6 mL microfuge tube containing 1 mL of 4% PFA for each spinal cord/mouse. Also prepare 15 mL of PBS per mouse.
29. Euthanize mouse with ketamine/xylazine overdose via intraperitoneal injection.
30. Perfuse the mouse via cardiac puncture.
    a. Disinfect the mouse with 70% ethanol.
    b. Lay the mouse on its back, use dissection scissors to make an incision around the middle abdomen to expose the diaphragm.
    c. Remove the right kidney of the mouse.
    d. Cut open the diaphragm to access the heart.
    e. Insert a 15 mL syringe with $26^{1}/_{2}$ gauge needle containing 15 mL of PBS into the right ventricle of the heart.
    f. Push the syringe slowly and perfuse the mouse with ~15 mL of PBS. The lungs and the liver should turn pale/white with appropriate perfusion.

31. Dissect and remove the spinal cord.
    a. Turn the mouse to lay on its front, pin its nose, arms, and legs to a dissecting board so the mouse's head and spinal cord are lying flat.
    b. Remove the skin from the upper lumber area to the head. Also cut to remove excess muscles and fat alone the back and arms.
    c. Cut through the skull from the front end, then on both side through the top of the skull using dissecting scissors.
    d. Using iridectomy scissors, carefully cut and remove the dorsal skull and vertebrae to expose the brain and spinal cord (Figure 3).

Note: If brain and spinal cord appear reddish, additional perfusion is needed.

    e. Gently lift the brain up with thick forceps and cut along both side of the spinal cords to free it from the vertebra column (Figure 3, **green dashed lines**).
    f. Trim the spinal cord to collect the lower cervical and thoracic regions containing the lesion site (Figure 3, **white dashed lines**).

32. Place the spinal cord piece on a segment of thick filter paper of equivalent width and length and submerge the tissue into 4% PFA in the 1.6 mL microfuge tubes for 8–12 h fixation at 4 C.
33. The next day, transfer the spinal cord tissue into 1.6 mL microfuge tubes containing 1 mL of 30% sucrose solution (30 g of sucrose dissolved in ddH_2O to a final 100 mL volume). Incubate in 30% sucrose solution for at least 48 h.

Note: This step dehydrates the tissue to prevent water crystal formation during freezing that can significantly damage the tissue. When the tissue is completely dehydrated, it will sink to the bottom of the tube instead of floating.

Optional: Experimental blinding may be performed at this step by asking a colleague to relabel and record the sample identities which will be unmasked after data acquisition and analysis.

34. Freeze spinal cords in cryo embedding molds.

Note: Freeze control (i.e. microglia sufficient) and treatment (i.e. microglia deficient) tissues together in the same mold so they may be stained and analyzed better consistency.

a. Submerging the spinal cord segments in molds containing FSC 22 Frozen Section Media (Leica).
b. Align spinal cords evenly in parallel.
c. Transfer the mold with tissues onto a dewar containing dry ice and isopentane, cover until it is a completely frozen tissue block.

Pause point: Frozen tissue blocks may be stored at −20°C for a few days to a week before cryosectioning. For long term storage, they can be stored at −80°C.

35. Serially cut spinal cord tissue blocks coronally using a cryostat and collect the tissue sections on to Superfrost Plus microscope slides (VWR) (Figure 4).

Note: If spinal cords were stored at −80°C, warm them up to −20°C in the cryostat before cutting.

a. Label 20 microscope slides appropriately with experimental parameters.
b. Cut the tissue block with spinal cords coronally into 20 μm sections sequentially onto slides starting at slide #1 and continue to slide #20.
c. Repeat until all spinal cord lesions have been captured onto slides.
d. Coronal sections (in columns going down) on the slide for each spinal cord are separated by 400 μm.

Pause point: Cut tissue sections on microscope slides can be stored at −20°C for at least 6 months, or at −80°C for longer prior to analysis.

Immunostaining spinal cord tissue sections

Timing: 3–5 days

Spinal cord lesion can be stained with various antibodies and analyzed by confocal microscopy to assess OxPC deposition, inflammation, neurodegeneration, and demyelination. For example, the E06 antibody (mouse monoclonal IgM, Sigma/Avanti) detects OxPC deposition, CD68 (rat monoclonal IgG, Biolegend) and IBA1 (rabbit polyclonal IgG, Wako Fujifilm) antibodies detect microglia/macrophages, neurofilament heavy chain (rabbit polyclonal IgG, Encor Biotechnology) detects axons and neurodegeneration, and myelin basic protein (rat IgG, Abcam) detects myelin and its disruption.

Optional: Demyelination and tissue cellularity in the lesion containing spinal cords can be assessed histologically using eriochrome cyanine and neutral red. Briefly, treat spinal cord

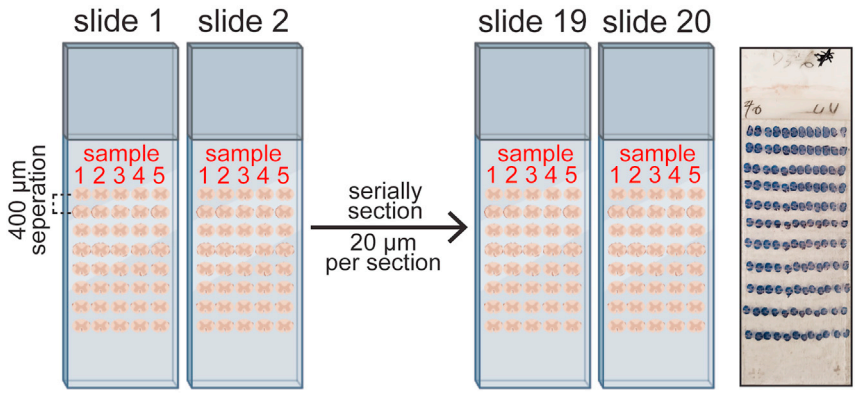


Figure 4. Schematic showing the setup for collecting cryosectioned spinal cord tissue sections

Picture on the right shows a representative slide containing spinal cord sections stained with eriochrome cyanine and neutral red. Figure partially made with Biorender.

tissues successively with citrisolv (Thermo Fisher), isopropanol, 100%, 95%, 90%, 70%, and 50% ethanol, 1 min each. Then wash with distilled water for 1 min, followed by incubation in eriochrome cyanine staining solution (10% FeCl3) for 15 min. Then, wash with ddH_2O for 1 min and with 0.5% NH_4OH for 5–10 sec, followed by 1 min of ddH_2O. Then incubate tissues with 1% neutral red solution for 2 min and wash ddH_2O water for 1 min. Dehydrate the tissues with successive 1 min washes of 50%, 70%, 90%, 95% and 100% ethanol, 2 min wash of isopropanol, 4 min of citrisolv, and mount the slide with coverslips using Acrytol mounting medium (Electron Microscopy Science). Use a brightfield microscope or slidescanner (i.e. 20× 0.75 NA air objective on the Olympus VS110 Slidescanner) to acquire images of serial spinal cord lesions. Using appropriate software such as ImageJ (NIH) or cellSens (Olympus), analyze demyelinated areas demarcated by lower eriochrome cyanine staining and higher neutral red staining in the ventral spinal cord white matter for lesion size and volume.

36. Thaw spinal cord tissue slides to 22°C–24°C and air dry (troubleshooting 7).
37. Wash and rehydrate by submerging tissues side in PBS for 10 min at 22°C–24°C.
38. Gently tap to dry the slides, place them flat on a staining apparatus and permeabilize the spinal cord sections by covering with 800 µL of PBS containing 0.2% Triton X-100 for 10 min at 22°C–24°C.
39. Gently tap to dry the slides, cover the tissue sections with 800 µL of horse blocking solution (PBS, 10% horse serum, 1% BSA, 0.1% cold fish stain gelation, 0.1% Triton X-100, 0.05% Tween-20) for 1 h at 22°C–24°C.

Note: If using mouse-derived antibodies for staining, add rat anti-CD16/32 Fc blocking antibody (BD Biosciences) at 5 µg/mL to the blocking solution to minimize non-specific immune cell Fc receptor binding to mouse antibodies.

40. Wash the slides once by briefly submerging in PBS and gently tap dry.
41. Cover the tissue sections with 600 µL of primary antibodies resuspended in antibody dilution buffer (PBS, 1% BSA, 0.1% cold fish stain gelation, 0.1% Triton X-100) for 8–12 h at 4°C.

Note: Ensure there is no overlap in the hosts of the primary antibodies. If the tissues were blocked with the rat anti-CD16/32 Fc blocking antibody, additional rat antibodies cannot be used. However, CD16/32 is a good marker for microglia/macrophage and monocytic cells in the spinal cord lesions. When staining tissues for the first time or with previously untested antibodies, isotype controls should be included to assess non-specific primary antibody binding.

42. Wash three times with PBS containing 0.2% Tween-20 for 5 min each at 22°C–24°C.
43. Cover the tissue sections with 600 μL of secondary antibodies and DAPI (1 μg/mL) resuspended in antibody dilution buffer for 1 h at 22°C–24°C.

Note: A slide stained with only the secondary antibodies and DAPI should be included to assess non-specific secondary antibody binding.

44. Wash three times with PBS containing 0.2% Tween-20 for 5 min each at 22°C–24°C.
45. Mount slides with coverslips using Fluoromount-G solution (SouthernBiotech). Air dry slides at 22°C–24°C for ~1 h.

Pause point: Stained slides may be stored in the dark at 4°C for at least 2 weeks priors to microscopy analysis.

46. Acquire z-stacks of the lesion epicenters for each spinal cord/mouse using a laser confocal microscope with appropriate lasers and detectors needed for the labeled secondary antibodies and DAPI in step 45 (troubleshooting 8).
    a. For our studies, we used a Leica TCS Sp8 laser confocal microscope with a 25X 0.5 NA water objective; 405 nm, 488 nm, 552 nm, and 640 nm excitation lasers; and two low dark current Hamamatsu PMT detectors as well as two high sensitivity hybrid detectors.
    b. Images were acquired in 8-bits, in a z-stack using uni-directional scanning, 1 times frame averaging, 1 airy unit pinhole, 0.75× zoom, and 0.57 μm per optical section and 2048 × 2048 pixels xy resolution.

Note: 0.57 μm per optical section was selected as it is the z-resolution at Nyquist using the 25X 0.5 NA water objective on the Sp8. The xy resolution may be increased to Nyquist however the amount of time required to image each sample will drastically increase.

    c. Save all raw data in Tiffs and project files for analysis and record keeping.

⚠ CRITICAL: Equal acquisition setting state above as well as equal laser, gain, and offset settings must be applied for all samples within each set of experiments. To minimize variability, stain and image multiple slides at once.

EXPECTED OUTCOMES

OxPCs were previously described as end-product reporter for oxidative damage and free radical formation in MS lesions (Haider et al., 2011). We recently demonstrated that OxPCs found in MS lesions are mediators of neurodegeneration and that microglia deficiency exacerbates OxPC induced neurodegeneration (Dong et al., 2021). Here, we present protocols for *in vitro* and *in vivo* approaches to study microglia functions in response to OxPC mediated neurodegeneration.

For primary neuron isolation from the brain cortices of mouse embryos, expected yield per brain is approximately 5×10^6 cells. Healthy neurons isolated from the brain cortices of mouse embryos will express tubulin β3 and grow interconnected processes after 2–3 days (Figure 5). In culture in 96-well plates, these neurons may be used as a high throughput assay to study neurodegeneration and neuroprotection by various compounds and molecules (Dong et al., 2021; Faissner et al., 2017). For microglia isolation from the brains of adult mice, expected yield per brain is approximately 0.5 to 1×10^6 cells. Microglia isolated from the adult mouse brain are $CD68^+$ and $Tmem119^+$ for at least 48 h in culture (Figure 6), and the addition of these cells in coculture with neurons makes this a useful model to assess how microglia may interact with neurons. In addition, the described method of adult microglia isolation has been tested with 6-week-old and 52-week-old mice, potentially allowing for comparisons on the effect of aging on microglia ex vivo and *in vitro*. Without perturbations, neurons

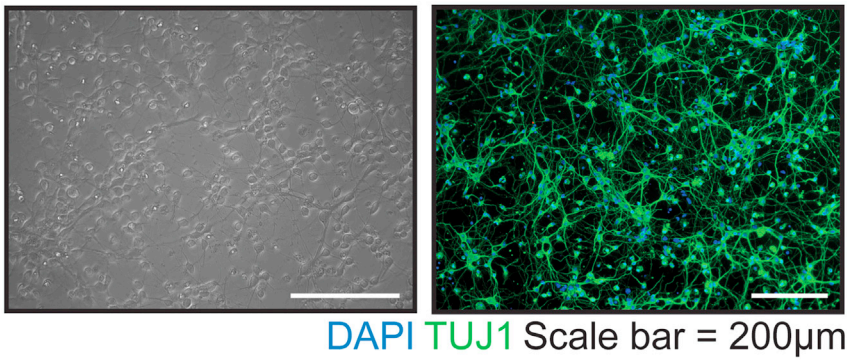


Figure 5. Representative microscopy image of healthy neurons with process outgrowth
Left image shows neurons by brightfield microscopy. Right composite image shows PFA fixed neurons labeled with DAPI (blue) and tubulin β3 (green). Scale bar: 200 μm.

cocultured with microglia at roughly 4:1 ratio (1×10^5 neurons to 2.5×10^4 microglia per well in a 96 well plate) remains healthy (Figure 7), potentially up to 3 weeks (data not shown). More importantly, brain derived microglia prevented the loss of tubulin $\beta3^+$ neurons after 24 h of OxPC treatment (Figure 8) while also inhibiting OxPC mediated upregulation of cleaved caspase 3 (Dong et al., 2021). Overall, this approach is a useful *in vitro* platform for screening molecules that may interfere or enhance the neuroprotective functions of microglia.

Injection of purified OxPCs (POVPC or PAzePC) into the ventral spinal cord white matter of mice induces a demyelinating lesion with $IBA1^+$ microglia/macrophage recruitment and accumulation, $E06^+$ OxPC deposition (progressively increase from day 1 to day 7 after injection), early depletion of oligodendrocytes (1 day after injection) followed by $OLIG2^+$ oligodendrocyte progenitor cell recruitment (7 days after injection), and loss of neurofilament heavy chain $(NFH)^+$ axons (Dong et al., 2021). Microglia depletion by DT injection in $CX3CR1^{CreER}$:$Rosa26^{iDTR}$ mice led to a larger lesion with increased $E06^+$ OxPC deposition as well as reduced microglia, $OLIG2^+$ cells, and NFH^+ axons compared to PBS injected $CX3CR1^{CreER}$:$Rosa26^{iDTR}$ mice (Figure 9) (Dong et al., 2021). While we tested microglia depletion for the first 7 days and between days 7 and 14 following OxPC injection, additional time points of microglia depletion will be of interest for future investigations. In addition, microglia depletion in $CX3CR1^{CreER}$:$Rosa26^{iDTR}$ mice of different age will help describe how aging impacts microglia functions. Thus, this approach allows for the temporal study of microglia in response to OxPC mediated neurodegeneration in the spinal cord white matter of mice.

QUANTIFICATION AND STATISTICAL ANALYSIS

1. Use multiwavelength cell scoring analysis function in the MetaXpress High-Content Image Acquisition and Analysis Software (Molecular Devices) to quantify neuron survival in cell culture experiments.

   Note: ImageJ (NIH) may also be used to quantify neuron survival, though this may be time costly without an automated macro script depending on the number of experimental conditions and replicates.

   a. Set DAPI channel as W1 to quantify total number of cells.
   b. Set tubulin β3 in Alexa Fluor 488 as W2 from W1 to quantify the number of $DAPI^+$ tubulin $\beta3^+$ neurons.

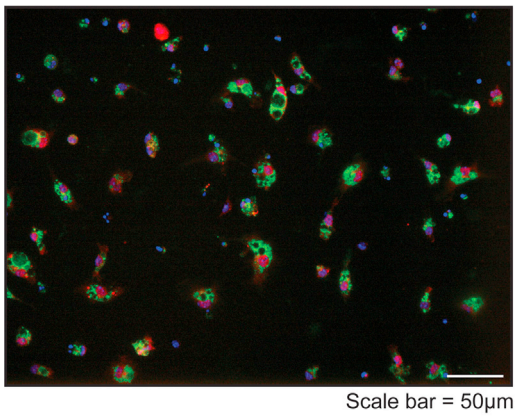


Figure 6. Composite immunofluorescence microscopy image of microglia isolated from the brains of adult mice after 48 h culture in neuron growth media

Blue – DAPI, Red – Tmem119, Green – CD68. Scale bar: 50 μm.

c. Use ordinary one-way ANOVA, Dunnett's multiple comparisons test to compare statistical differences in the total number of survive neurons between experimental conditions.
d. Visualize representative images from different conditions by merging and displaying each acquired channels (DAPI, Alexa Fluor 488, Alexa Fluor 647) in pseudo colors using ImageJ (NIH).

2. Use ImageJ to analyze and compare each z-stack confocal images of spinal cord lesion epicenters (Figure 10).
   a. Import z-stack. Tiff image sequences for each channel/marker for a spinal cord sample.
   b. Create maximum intensity projections for each channel/marker and convert the images from 8-bit to RGB. Save these images for analysis and record keeping.
   c. Draw a region of interest around the OxPC induced lesion and clear the outside.

Note: Depending on the staining set, different markers such as DAPI (increased cellularity in the lesion), CD68/IBA1 (microglia/macrophage accumulation in the lesion), or myelin basic protein (myelin disruption in the lesion) may be used to demarcate the lesion area.

   d. Determine the positive signal using the color brightness threshold.

Note: A slide stained with secondary antibody alone, or a normal appear tissue is useful here to distinguishing negative/baseline signals from positive signals.

   e. Use the analyze particles function to quantify the positive signals in each region of interest. Record the raw values for analysis and record keeping.

⚠ CRITICAL: Recording and using the same threshold values (color for positive signals, size, and circularity for particle analysis) for all samples is a must to avoid bias and to maintain consistency in the analysis.

   f. Use unpaired two-tailed t-test to compare experiments with 2 groups, or use ordinary one-way ANOVA, Dunnett's multiple comparisons test to compare experiments with more than 2 groups.

Note: Use statistical test for normality such as the Kolmogorov-Smirnov test to determine if the data are normally distributed and whether statistical tests are appropriate.

   g. For representative images, merge and display maximum intensity projection of each channel/marker in a z-stack using pseudo colors in ImageJ.

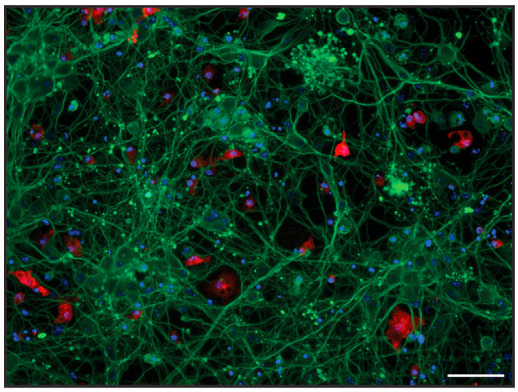


Figure 7. Composite immunofluorescence microscopy image of primary mouse microglia and neurons in coculture after 48 h

Blue – DAPI, Red – IBA1, Green – tubulin β3. Scale bar: 50 μm.

Note: Adjustments to brightness and contrast for representative images should be consistent across all samples.

LIMITATIONS

Neurons

The *in vitro* neuron microglia coculture model uses neurons derived from the brain cortices of embryos between embryonic gestation days 14 and 16. These neurons likely do not represent adult neurons found in the brain and spinal cord. However, adult brain-derived neurons have not survived the isolation process in our experience. Thus, potential interactions between mismatched adult derived microglia and embryo derived neurons *in vitro* will require careful testing *in vivo*.

Microglia isolation and purity

The yield of microglia isolated using this protocol is limited per mouse brain. Thus, pooling multiple brains for microglia isolation is recommended. Additionally, the commercial kit used for microglia enrichment uses $CD11b^{+}$ antibody positive selection and thus present two potential limitations: 1) non-microglial myeloid cells such as monocytes or border associated macrophages may be included from processing the whole brain and 2) CD11b antibody remaining on isolated cells may lead to abnormal cellular response/activation.

OxPC spinal cord injection

While using non-oxidized DPPC is recommended as a negative control for *in vitro* cell culture experiments, DPPC cannot be injected into the spinal cords of mice as it does not dissolve in aqueous solutions. Given DPPC does not harm neurons *in vitro* and PC species are found in high abundance in myelin and as components of cell membranes, we do not expect DPPC to induce neurodegeneration. Nevertheless, the effect of excess PC in the spinal cord white matter has not been tested and remains unclear. Moreover, the acute demyelination and neurodegeneration induced by this method are relatively severe and thus it may be difficult to achieve protective effects when testing potential therapeutics. Thus, varying the amount of OxPC injected into the spinal cord may be required.

Microglia depletion

The CX3CR1 promoter is also active in meningeal macrophages, choroid plexus macrophages, and perivascular macrophages of the central nervous system (Masuda et al., 2020). Using $CX3CR1^{CreER}$:$Rosa26^{iDTR}$ mice will likely deplete microglia in addition to these border-associated macrophages. Thus, future studies should also consider delineating potential functional differences between microglia and border associated macrophages. Microglia depletion in this protocol is throughout the central nervous system and not restricted to the spinal cord lesion, thus how tissue

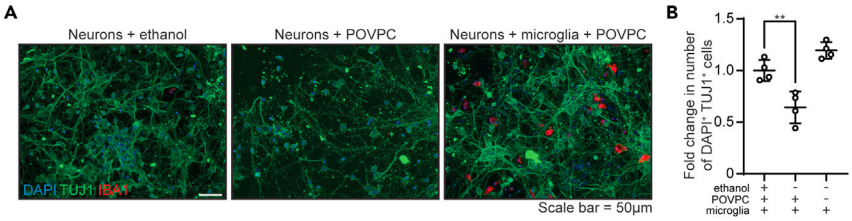


Figure 8. OxPC neurotoxicity is mitigated by microglia in culture

(A) Representative composite widefield microscopy images of mouse neurons cocultured with or without adult brain derived microglia, labeled with DAPI for nuclei, Tuj1 for tubulin β3, and IBA1 (or Tmem119 or P2ry12) for microglia, 24 h after treatment with ethanol control or POVPC. Scale bar: 50 μm.

(B) Bar graph comparing the fold change in the number of $DAPI^+$ $Tuj1^+$ neurons after ethanol or POVPC treatment for 24 h. Data are represented as mean ± SD. Significance indicated as ** $p < 0.01$, one-way ANOVA comparing the treatments against the ethanol alone control.

wide microglia deficiency affect tissue homeostasis and whether this impact the OxPC lesion needs additional investigation.

TROUBLESHOOTING

Problem 1

Poor neuron survival and health (step 4).

Potential solution

Thoroughly wash to remove the polyornithine from tissue culture flasks or 96-well plates. Make sure the water bath temperature does not exceed 38°C when dissociating the cells. Avoid vortexing and harsh pipetting when dispersing cell pellets into a single cell suspension. Check that the neuron growth medium has not expired. Only exchange half the cell culture medium at any time and do not expose neurons to air. Check that the cell culture incubator has the correct settings.

Problem 2

High amounts of debris found in cells isolated from the adult mouse brain (step 8).

Potential solution

Ensure centrifuge break settings are in accordance with the protocol. Take extra care when pipetting to remove the myelin layer and the supernatant layer after Percoll centrifugation steps.

Problem 3

Purified OxPCs do not consistently cause neuron damage (step 11).

Potential solution

Purified OxPCs such as POVPC and PAzePC are supplied as lyophilized powders from the manufacturer and at time may be difficult to see in the glass vials. Carefully wash the bottom and all sides of the glass vial to ensure full resuspension of OxPCs into solution. OxPCs will hydrolyze and degrade in aqueous solution. Thus for *in vitro* experiments resuspend OxPCs in 100% ethanol and dilute with neuron growth medium just prior to experiment. For *in vivo* experiments, resuspend OxPCs in PBS just prior to spinal cord injections. Minimize the amount of time that OxPCs are resuspended in buffers.

Problem 4

Spinal cord injection did not produce lesions (step 24).

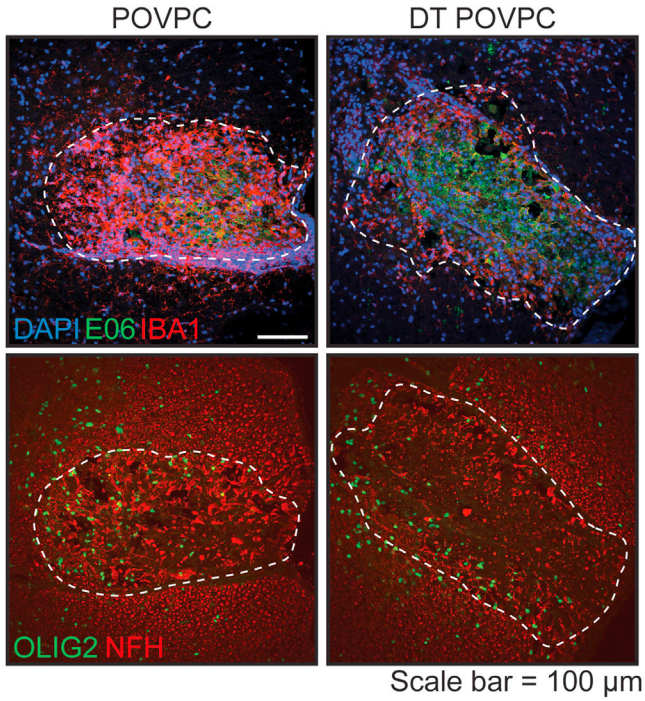


Figure 9. Microglia deficiency exacerbates OxPC-induced neurodegeneration

Representative composite confocal microscopy images of spinal cord lesions in CX3CR1^{CreER}:Rosa26^{iDTR} mice with PBS (microglia sufficient) or with DT injection (microglia depletion) for 7 days after OxPC injection. Top panel shows spinal cord sections labeled with DAPI for nuclei, E06 for OxPC, and IBA1 for microglia/macrophages (alternatively CD68 and Tmem119 or P2ry12 may be used to differentiate microglia from peripheral monocytes and monocyte derived macrophages). Lower panel shows spinal cord sections labeled with OLIG2 for oligodendrocyte progenitor cells and NFH (neurofilament heavy chain) for axons. Scale bar: 100 μm. Dashed lines demarcate the OxPC lesion.

Potential solution

Ensure that the meninges covering the dorsal spinal cord are removed before inserting the Hamilton needle into the spinal cord. The tip of the needle may appear bent if it is not properly inserted. Ensure the needle is inserted off center to the right side of the midline so the injection does not erroneously occur through the ventral median fissure of the spinal cord.

Problem 5

Open sutures are found in mice after spinal cord surgery (step 25).

Potential solution

Use interrupted sutures or staples for closing the wound on the back.

Problem 6

Insufficient microglia depletion (step 26).

Potential solution

Check that the genotype of the CX3CR1^{CreER}:Rosa26^{iDTR} mice is correct. This may be done using PCR according to instructions from JAX (For CX3CR1^{CreER} see https://www.jax.org/Protocol?stockNumber=021160&protocolID=24463; for Rosa26^{iDTR} see https://www.jax.org/Protocol?stockNumber=007900&protocolID=22285). Alternatively, CX3CR1^{CreER} expression may be validated by EYFP expression and Rosa26^{iDTR} expression may be validated by an anti-DTR antibody. Also check that the DT used was reconstituted and stored appropriately and within the expiration date.

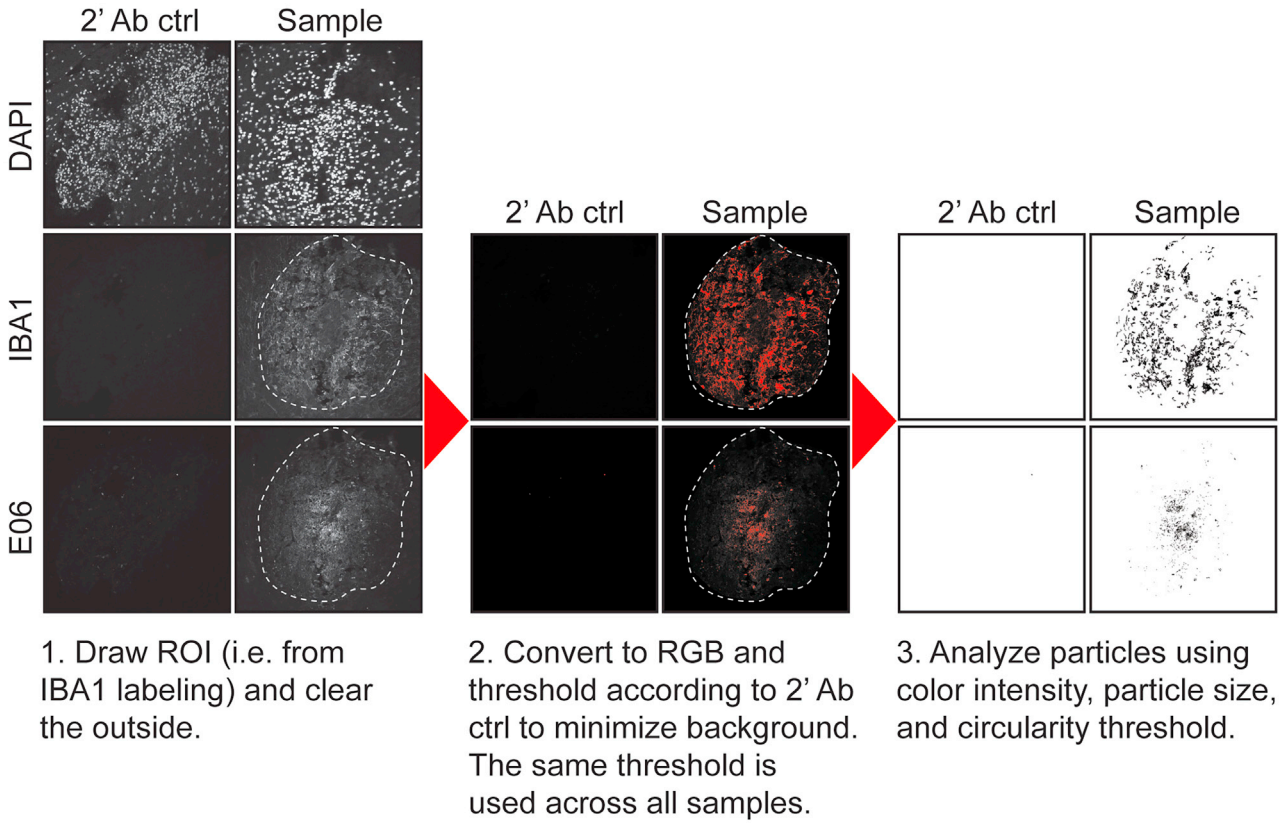


Figure 10. Flowchart of quantitative confocal microscopy analysis

Problem 7

Spinal cord sections contain holes formed from water crystals throughout the tissue (step 36).

Potential solution

Check that fixation and dehydration are properly completed. Properly fixed and dehydrated spinal cords should be rubbery and should sink in 30% sucrose solution. Warm and air-dry microscope slides with spinal cord sections at 22°C–24°C before proceeding to staining steps.

Problem 8

High background signals from immunofluorescence (step 46).

Potential solution

If using mouse derived primary antibodies, ensure that Fc receptors are blocked. Check antibody specificity to the tissue sections with appropriate isotype controls, secondary antibody controls, and sham/normal tissue controls. Check the laser power, gain, and offset are appropriately set and consistently for image acquisition.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. V. Wee Yong (vyong@ucalgary.ca).

Materials availability

This protocol is not associated with any new unique reagents.

Data and code availability

The published article includes all datasets generated or analyzed during this study.

ACKNOWLEDGMENTS

We thank the Hotchkiss Brain Institute Advanced Microscopy Platform Facility for microscopy help. The authors' research is supported by operating grants from the Alberta Innovates Health Solutions CRIO Team program, the MS Society of Canada, and the Canadian Institutes of Health Research. Y.D. acknowledges postdoctoral fellowship support from the Canadian Institutes of Health Research and Alberta MS Collaboration. B.M.L. gratefully acknowledges studentships from the Alberta Graduate Excellence Scholarship and the MS Society of Canada Graduate Scholarship. V.W.Y. acknowledges salary support from the Canada Research Chair (Tier 1) program.

AUTHOR CONTRIBUTIONS

Y.D. conceived the project; designed, performed, and analyzed experiments; and wrote the first draft of this protocol. B.M.L. was key for mouse spinal cord surgeries and its associated protocol steps. C.S. was key for the primary mouse neuron protocol. V.W.Y. co-conceived the project, provided support and experimental design, supervised the overall study, and critically edited the manuscript. All authors reviewed and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Dong, Y., D'Mello, C., Pinsky, W., Lozinski, B.M., Kaushik, D.K., Ghorbani, S., Moezzi, D., Brown, D., Melo, F.C., Zandee, S., et al. (2021). Oxidized phosphatidylcholines found in multiple sclerosis lesions mediate neurodegeneration and are neutralized by microglia. Nat. Neurosci. *24*, 489–503.

Faissner, S., Mishra, M., Kaushik, D.K., Wang, J., Fan, Y., Silva, C., Rauw, G., Metz, L., Koch, M., and Yong, V.W. (2017). Systematic screening of generic drugs for progressive multiple sclerosis identifies clomipramine as a promising therapeutic. Nat. Commun. *8*, 1990. https://doi.org/10.1038/s41467-017-02119-6.

Haider, L., Fischer, M.T., Frischer, J.M., Bauer, J., Hoftberger, R., Botond, G., Esterbauer, H., Binder, C.J., Witztum, J.L., and Lassmann, H. (2011). Oxidative damage in multiple sclerosis lesions. Brain *134*, 1914–1924.

Masuda, T., Amann, L., Sankowski, R., Staszewski, O., Lenz, M., D Errico, P., Snaidero, N., Costa Jordao, M.J., Bottcher, C., Kierdorf, K., et al. (2020). Novel Hexb-based tools for studying microglia in the CNS. Nat. Immunol. *21*, 802–815.