Protocol

Protocol for brain-wide or region-specific microglia depletion and repopulation in adult mice

1. Oral gavage CX3CR1creERT2 x iDTR- mice with tamoxifen (5 days)

2. Turnover of CX3CR1 positive cells in the blood and bone marrow (4-6 weeks)

3a. DT intra-peritoneal injections (3 days)

3b. DT intra-hippocampal injection (~30 min)

The advent of tools enabling the direct manipulation of microglia has furthered our understanding of their role in health and disease. Here, we present a detailed protocol allowing for microglia turnover in adult CX3CR1creERT2 x iDTR or CX3CR1creERT2 x iDTR x tdTomato<sup>lox/lox</sup> mice, either in a brain-wide or region-specific manner, and their subsequent isolation for downstream applications. This protocol may be used to explore microglia biology and their putative region-specific heterogeneous functional diversity, expanding our understanding of their importance in various neuroinflammatory conditions.

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HIGHLIGHTS

- Conditional genetic depletion and/or repopulation of microglia from adult mouse brain
- Targeted ablation of hippocampal microglia
- Adaptable for region-specific depletion of microglia
- FACS isolation of microglia from the adult mouse hippocampus

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Protocol for brain-wide or region-specific microglia depletion and repopulation in adult mice

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SUMMARY
The advent of tools enabling the direct manipulation of microglia has furthered our understanding of their role in health and disease. Here, we present a detailed protocol allowing for microglia turnover in adult CX3CR1creERT2iDTR or CX3CR1creERT2iDTRtdTomatoflox mice, either in a brain-wide or region-specific manner, and their subsequent isolation for downstream applications. This protocol may be used to explore microglia biology and their putative region-specific heterogeneous functional diversity, expanding our understanding of their importance in various neuroinflammatory conditions.

For complete details on the use and execution of this protocol, please refer to Willis et al. (2020)

BEFORE YOU BEGIN
Sensitizing mice to diphtheria toxin-induced turnover
For microglia turnover, we utilize CX3CR1creERT2iDTR or CX3CR1creERT2iDTRtdTomatoflox mice, see Key resources table with The Jackson Laboratory stock numbers. Generation of CX3CR1creERT2iDTR mice is detailed within Parkhurst et al. (2013). The CX3CR1creERT2iDTR mice are heterozygous for the CX3CR1creERT2 and homozygous for the iDTR gene. As both the iDTR and the tdTomato genes are expressed through the Rosa26 reporter, CX3CR1creERT2iDTRxtdTomatoflox mice are heterozygous for CX3CR1creERT2, heterozygous for iDTR, and heterozygous for tdTomato. In these mice, induction of the diphtheria toxin receptor (DTR) expression in CX3CR1-expressing cells is achieved by tamoxifen treatment driving Cre recombinase induced recombination. The induction of DTR expression in CX3CR1-expressing cells sensitizes these cells to diphtheria toxin (DT) induced cell death. As CX3CR1-expressing cells also include peripheral monocytes, a rest period of approximately 4–6 weeks is necessary to allow for monocyte turnover, leaving the longer-lived microglia sensitive to DT-induced cell death (Willis et al., 2020; Parkhurst et al., 2013; see Limitations).

Mouse colonies are bred and maintained in house in a University of Queensland Biological Resources’ specific pathogen-free “behind barrier” facility. Mice are housed socially (3–5 mice per cage) on a 12 h light/dark cycle in individually ventilated cages with ad libitum access to food and water. All experiments are conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes, with approval from the University of Queensland animal ethics committee. Refer to the Key resources table for a complete list of materials and equipment.
Oral gavage CX₃CR₁<sup>creERT2</sup> × iDTR mice or CX₃CR₁<sup>creERT2</sup> × iDTR × tdTomato<sup>flox</sup> mice

Timing: 5 days (to be performed at least 4–6 weeks prior to diphtheria toxin administration)

Oral gavage the CX₃CR₁<sup>creERT2</sup> × iDTR mice or CX₃CR₁<sup>creERT2</sup> × iDTR × tdTomato<sup>flox</sup> mice at four- to six-weeks of age once daily for five consecutive days at approximately the same time of day.

1. Tamoxifen preparation:
   a) Calculate the tamoxifen dose (12.5 mg/g body weight) based on the mice’s body weight, weighed shortly prior to the first round of oral gavaging. The mouse’s weight can be monitored during the course of the tamoxifen treatment, to ensure there is no weight changes that could impact the relative tamoxifen dose they receive.
   b) Prepare the tamoxifen solution (25 mg/mL; Sigma) in a biosafety cabinet by adding the tamoxifen crystals directly to corn oil (Sigma) in either a 2 mL Eppendorf tube or a 5 mL specimen jar. To more easily dissolve the tamoxifen crystals, the tamoxifen solution should be made in small volumes of no more than 3 mL. Prepare approximately 1 mL extra of tamoxifen solution than what is needed, to compensate for any loss during gavaging. Seal the tube by wrapping the lid with parafilm.
   c) Vortex the tube containing the tamoxifen solution and submerge in a sonicator water bath at 22°C–24°C for approximately 1 h, vortexing every 15 min until the tamoxifen is fully dissolved (the solution should be clear with no visible crystals).
   d) Keep the tamoxifen solution light protected by wrapping in aluminum until use. The tamoxifen can be kept short-term (>1 h) at 22°C–24°C; otherwise the tamoxifen solution can be kept at 4°C for approximately 24 h. The tamoxifen solution should be clear; if the solution becomes cloudy, the tamoxifen is coming out of solution and should be discarded.
   e) Prepare the tamoxifen solution fresh daily, just prior to use.

   △ CRITICAL: Care should be taken to the timings of tamoxifen administration and subsequent diphtheria toxin treatment as this will affect which cell population/s are depleted.

2. Oral gavaging:
   a) Use an appropriately sized gavage tube that is bulb tipped and flexible, e.g., plastic feeding tubes, 20ga × 38 mm (Instech Laboratories USA). The gavaging tube length should measure the distance from the mouth to just past the last rib of the rodent, to reach the rodent’s stomach.
   b) Load the gavaging syringe with the corn oil or tamoxifen solution.
   c) Attach the gavage tube, removing dead space and any air bubbles.
   d) Restrain the mouse so that the head and body are in line and the body remains in an upright position, allowing for easier gavage.
   e) Insert the gavaging tube into the side of mice’s mouth and advance down the esophagus to the required length of the gavage tube. If in the correct position, there will be a little resistance. You should not feel resistance when the needle is going down, if you do, you are probably going down the trachea and should withdraw and re-insert.
   f) Slowly press on the plunger to administer the solution, then gently and slowly withdraw the gavage tube. Doing this too fast may cause stomach reflux and the mouse may bite down or swallow the tubing and choke.
   g) For vehicle-treated mice, mice can be oral gavaged with plain corn oil, where the volume to be gavaged is calculated as per gram body weight as above.
h) Return the mouse to its home cage and leave to rest for at least 4–6 weeks to allow for the turnover of CX3CR1-positive cells (i.e., monocytes) in the blood and bone marrow.

**Note:** Tamoxifen can be delivered via other approaches, such as intraperitoneal (IP) injection. However, if delivering the tamoxifen or corn oil solution via IP injection, care should be taken as the oil may accumulate in the abdomen and negatively affect the mice’s wellbeing.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Tamoxifen           | Sigma-Aldrich | Cat# T5648 |
| Plastic feeding tubes, 20ga (yellow) × 38 mm, sterile | Instech Laboratories USA | Cat# FTP-20-38 |
| Diphtheria toxin    | Sigma-Aldrich | Cat# D0564-1MG |
| UltraPure RNase/DNase-free distilled water | Invitrogen | Cat# 10977023 |
| Corn oil            | Sigma-Aldrich | Cat# C8267-500ML |
| Sodium chloride 0.9% for injections (injectable saline) | Baxter | SKU: 2B1324X |
| Zoetil 100 (active constituents: 250 mg tiletamine and 250 mg Zoalazepam; 100 mg/mL final stock concentration) | Virbac | N/A |
| Xylazine-20 (20 mg/mL) | Ilium | N/A |
| Papain              | Worthington Biochemical Corporation | Cat# LS003120 |
| DMEM/F-12, HEPES    | Life Technologies | Cat# 11330057 |
| HBSS                | Gibco | Cat# 14175095 |
| Leibovitz’s L-15 medium | Gibco | Cat# 11415064 |
| DNase I             | Roche | Cat# 10104159001 |
| Penicillin-streptomycin | Sigma-Aldrich | Cat# P4333-20mL |
| Bovine serum albumin (BSA) solution | Sigma-Aldrich | Cat# A8412-100mL |
| Critical commercial assays | | |
| Quick RNA extraction Miniprep kit | Zymo Research Corp | Cat# R1054 |
| Experimental models: organisms/strains | | |
| Mouse: CX3CR1<sup>creERT2</sup> × iDTR | Parkhurst et al., 2013 | N/A |
| Mouse: CX3CR1<sup>creERT2</sup>; B6.129P2(C)-Cx3cr1tm2.1(cre/ERT2)Jung | The Jackson Laboratory | JAX: 020940, RRID: IMSR_JAX:020940 |
| Mouse: iDTR: ROSAiDTR | The Jackson Laboratory | JAX: 007900 |
| Mouse: CX3CR1<sup>creERT2</sup> × iDTR × tdTomato | Willis et al., 2020 | N/A |
| Software and algorithms | | |
| FlowJo version 10.4.2 | TreeStar | https://www.flowjo.com |
| Other | | |
| 40 μm cell strainer | Falcon | Cat# 734-0002 |
| Polyethylene tubing (30.5 m/100 ft; ID 0.38 mm/0.015 inch, OD 1.09 mm/0.043 inch) | BD Intramedic | Cat#427406 |
| Hamilton gastight syringe, volume 10 μL | Sigma | Model: 1701 HAM7653-01 Cat#HAM7653-01-1EA |
| Hamilton microliter syringe needle 26s gauge | Sigma | Cat#Z121401-6EA |
| Syringe pump | Harvard apparatus | N/A |
| Digital mouse stereotaxic instrument | Stoelting Company | Cat#51703D |
| Heat mat | N/A | N/A |
| BD Influx Cell Sorter | BD Biosciences | N/A |
| Vicryl dissolvable sutures | J&J, Ethicon | Cat#J493G |
### KEY RESOURCES TABLE

#### Papain dissociation buffer

| Reagent                                      | Final concentration | Amount                  |
|-----------------------------------------------|---------------------|-------------------------|
| Papain (Worthington Biochemical Corporation)  | 1 mg/mL             | 50 mL papain stock solution at 2 mg/mL concentration: add 100 mg of papain powder to 50 mL Leibovitz’s L-15 medium. Dissolve in a water bath at 37°C |
| HBSS without calcium and without magnesium    | –                   | 25 mL                   |
| DNase I (Roche)                               | 0.25 mg/mL          | 25 mL of DNase I stock solution at 1 mg/mL concentration: To make 1 mg/mL DNase I stock solution, add 25 mg DNase I powder to 25 mL DMEM/F-12, HEPES media |

### MATERIALS AND EQUIPMENT

#### Microglia isolation buffer

| Reagent                                      | Final concentration | Amount |
|-----------------------------------------------|---------------------|--------|
| Dulbecco’s modified Eagle medium (DMEM F12 with HEPES) | –                   | 97 mL  |
| BSA                                           | 2%                  | 2 mL   |
| Penicillin-streptomycin (Sigma-Aldrich)       | 1%                  | 1 mL   |

**Note:** Papain dissociation buffer can be made in batches and stored in aliquots of 5 mL at –20°C longer-term (6 months). Aliquots of papain dissociation buffer can be thawed when needed by placing in a water bath at 37°C.

**Note:** Microglia isolation buffer can either be made fresh or ~1 day prior to use and stored at 4°C.

### STEP-BY-STEP METHOD DETAILS

#### Inducing widespread depletion and repopulation of microglia

© Timing: 3 days

Here we detail how microglial depletion/repopulation can be induced by systemic diphtheria toxin (DT) administration via intraperitoneal (IP) injection into tamoxifen-treated CX3CR1<sup>creERT2</sup> x iDT mice or CX3CR1<sup>creERT2</sup> x iDT x tdTomato<sup>fox</sup> mice.

1. Diphtheria toxin (DT; Sigma-Aldrich) stock solution is prepared at 2 mg/mL in sterile water (e.g., UltraPure water), according to manufacturer’s instructions, and stored at –80°C in 5 μL aliquots. Avoid freeze thawing DT.
   a) The expiration date of the DT stock solution is lot-specific and can be found on the manufacturer’s Certificate of Analysis (Sigma-Aldrich).
2. Working solutions of DT (3 ng/μL) are made by adding 3 μL of DT stock solution to 2 mL of injectable saline in a 2 mL Eppendorf tube. Vortex the working solution and place on ice.
3. DT is injected at a dose of 30 ng per gram body weight with 10 μL per gram body weight volume injected; e.g., 20 g mouse will be injected with 200 μL of DT working solution for a 600 ng dose of DT.
   a) To inject working DT solution, mice are weighted and appropriate dose/volume DT working solution loaded into a 1 mL syringe attached to 25-gauge needle.
b) Restrain the mouse and inject IP with working DT solution. Return the mouse to its home cage.

c) Mice are injected once daily for three consecutive days, at approximately the same time of day. Microglia should be depleted (>90% depletion) for at least ~24 h after the last DT injection and will undergo endogenous repopulation thereafter. See Troubleshooting 1.

d) Monitor the mice’s weight during DT treatment. Mice should lose no more than 20% of their original body weight over the course of DT treatment. If they do lose greater than 20% of their original body weight, or become sick, then cease DT treatment and monitor mouse’s health.

⚠️ CRITICAL: Once diluted, DT working solution should be used within 1 h. Always keep DT on ice. Persons working with mice and DT should have a current diphtheria vaccination.

**Inducing region-specific depletion/repopulation of hippocampal microglia**

**Timing:** ~30 min per animal

Inducing microglial turnover can also be achieved in a region-specific manner for the hippocampus via stereotaxic intra-hippocampal injection of DT into tamoxifen-treated CX3CR1creERT2 × iDTR mice or tamoxifen-treated CX3CR1creERT2 × iDTR × tdTomato<sup>lox</sup> mice using stereotaxic rig (Figure 1).

4. Prepare the DT working solution to deliver 0.1 ng of DT in 0.5 μL of sterile saline. Prepare DT working solution just prior to use. Do not freeze-thaw DT.

5. Attach the Hamilton syringe to the syringe pump (Figure 2).

6. Fill the Hamilton syringe with sterile saline using a 1 mL syringe (with no needle attached).

7. Prepare the polyethylene tubing prior to surgery. Insert an 18-gauge needle head (needle only) to the end of the tubing. Secure the needle in place with nail polish. Allow nail polish to harden. UV the tubing and keep the needle sterile.

8. Prior to attaching to the Hamilton syringe, use an 18-gauge needle attached to 1 mL syringe loaded with sterile injectable saline and perfuse saline through the prepared tubing, ensuring that there are no leaks in the tubing and the saline is running through the needle.

9. Attach the open end of the polyethylene tubing to the Hamilton syringe needle, careful not to puncture holes in tubing. The tubing should be inserted onto the Hamilton syringe by about 0.5 cm to ensure a stable connection (Figure 3).
10. Connect the tubing with needle end to the stereotaxic rig and gently hold in place with the stereotaxic rig needle attachment. Be careful not to disturb the needle head or dislodge the needle from the tubing (Figure 4).

11. Press the plunger of the Hamilton syringe and expel the sterile saline in the tubing. Carefully remove the droplet from the needle using a sterile tissue or separate sterile 18-gauge needle head.

12. Draw up some air into the tubing then use a sterile 0.6 mL Eppendorf tube lid filled with DT working solution or sterile saline and draw up into the tubing via the needle head of the tubing (Methods Video S1). This will allow for a gap (marked with a sharpie) between the sterile saline from the Hamilton syringe and your loaded solution, making it easier to tell whether your solution is indeed be delivered and running through the tubing appropriately. Ensure that there are no bubbles when drawing up the working solution into the tubing (Figure 5).

13. Anesthetize the mice via IP injection of 0.01 mg/g BW tiletamine/zolazepam (Zoletil) and 0.01 mg/g BW xylazine. Zoletil/xylazine solution is prepared as below:
   a) Using sterile syringe, transfer 0.1 mL of xylazine stock into a 1.5 mL Eppendorf tube.
b) Add 0.1 mL Zoletil and mix in with 0.1 mL of xylazine by vortex.

c) Take 0.1 mL of the Zoletil/xylazine stock mixture and put in new 1.5 mL Eppendorf tube.

d) Add 0.9 mL of injectable saline and mix very well by vortex.

Figure 4. Tubing with needle end connected to Hamilton syringe held in place to stereotaxic rig

Figure 5. Tubing filled with working solution to be injected using stereotaxic setup
e) For dosing: use just under 0.1 mL/10 g. For example, for a 20 g mouse, give between 0.18–0.19 mL; for a 30 g mouse give 0.28 mL as a starter. Prepare Zoletil/xylazine mixture either day before surgery (kept in fridge at 4°C for ~8–24 h) or just prior to surgery.

14. Trim the fur on top of the mouse’s head where the surgery site will be and mount the mouse into the stereotaxic frame.

15. Coat the mouse’s head with betadine using a cotton tip.

16. Place eye drops or vaseline to mouse’s eyes to ensure they do not dry out during surgery.

17. Using a scalpel, make a mid-line incision (1.5 cm long) along the skull, and retract the skin using non-dissolvable sutures to keep the skin away from skull.

18. Use a surgical marker pen to mark the Bregma location on the skull.

19. Move the needle to be positioned directly above Bregma and zero the coordinates on the stereotaxic. Raise the needle, so that you do not hit or scrape the skull when moving the needle and move the needle to the required coordinates (X, −2.0 mm; Y, −1.50 mm). Mark this location with the surgical marker. This will be the site to be injected.

20. Carefully and gently drill out a small hole where the injection site will be. Remove the skull piece carefully with fine forceps. Take great care to not damage the underlying cortex.

21. Lower the needle to the surface of the cortex. Zero the Z coordinates.

22. Carefully and slowly lower the needle into the brain over 2 min until you reach -2.0 mm Z coordinates.

23. Let the needle rest for 3 min.

24. Start the syringe pump to deliver the solution. Use a flow rate of 0.2 μL/min. For DT we deliver 0.1 ng in 0.5 μL over 2.5 min.

25. Stop the pump and allow to rest for 3 min.

26. Slowly draw up the needle. Advance 0.1 mm upward every 30 s for the first 1 mm, then slowly draw up for the remaining 1 mm over another 2–3 min.

27. Remove the mouse from the stereotaxic frame and place mouse on a heat mat to maintain the animal’s core body temperature.

28. Suture the incision.

a) Any type of sutures can be used. We typically use coated Vicryl dissolvable sutures (J493G, J&J, Ethicon).

29. Monitor the mouse until it has recovered and is awake and walking normally. Return mouse to home cage. Mice should be monitored daily to ensure it has successfully recovered from surgery and the sutures stay intact and the incision site heals.

Note: The coordinates used herein may be adapted to target other brain regions of interest. Here, with the coordinates above we have targeted microglia in the dorsal hippocampus.

Fluorescence activated cell sorting of microglia

© Timing: ~1 h 45 min per animal

Below we detail how to isolate resident or repopulated microglia (tdTomato-positive) from the hippocampus of tamoxifen-treated CX3CR1creERT2 × iDTR × tdTomato^flox^ mice. Mice with resident microglia are administered saline, while DT is administered to induce depletion and endogenous microglia repopulation, as detailed above See Troubleshooting 1. The efficiency of the microglial depletion and repopulation can be assessed by comparing numbers of microglia in microglial depleted animals with vehicle-treated controls (i.e., mice with resident microglia), either via fluorescence activated cell sorting (detailed below), or by immunostaining fixed brain sections with microglial markers (e.g., Iba1).

30. Mice are euthanized by cervical dislocation and the brains rapidly dissected from skull.
31. Remove the brain and submerge in ~3 mL of pre-chilled “microglia isolation buffer” in 50 mL falcon tube on ice. An exact volume of pre-chilled “microglia isolation buffer” is not necessary, so long as the brain is fully submerged in the buffer.

32. In laminar flow hood, transfer brain onto petri dish with drops of ice-cold isolation media (approximately 100–200 μL), and quickly dissect out hippocampus (Figure 6).
   a) Use surgical blade and cut along optic chiasm to remove rostral region of septo-diencephalon (viewed from bottom of brain)
   b) From the top of the brain, use forceps to separate the hemispheres, and push the hemisphere aside.
   c) Using circular/rolling motion, raise the hippocampus from overlying cortex and carefully remove using curved forceps. Using the forceps, transfer the isolated hippocampus to a separate petri dish.
   d) Keep this isolated hippocampus in drops of ice-cold DMEM F12 with HEPES (~200 μL) in a petri dish on ice until remaining dissections are complete.
e) Repeat for other hemisphere and/or other brain/s. Use a separate petri dish per sample and keep on ice. Process at most 2 mice/samples per run to ensure that all processing is done as quickly as possible. Samples from multiple mice can be pooled to isolate enough cells for downstream applications.

33. Using a scalpel blade, gently dice hippocampi in petri dish on ice until no obvious lumps of tissue remain.

34. Add 0.5 mL of the “papain dissociation buffer” per hippocampi, 0.25 mL onto the minced hippocampi and 0.25 mL on to the side of the petri dish (Figure 7). Use a 1 mL pipette to aspirate papain and hippocampi and transfer into a 15 mL Falcon tube. Use the extra papain solution on the side of the petri dish to collect any leftover cells/tissue before adding to the same falcon tube.

35. Place the tubes into the 37°C water bath for 8 min.

36. Remove the tubes from the water bath and using a 1 mL pipette very gently titrate (i.e., pipette up and down) the mixture 3 times by holding the tip of the pipette against side of the tube with some pressure. This step is important to break down any clumps, producing a single cell suspension (Methods Video S2).

37. Return tubes to water bath for another 8 min.

38. Gently triturate 2 times as above.

39. Gently add 3–4 mL of warm isolation buffer to tube by adding to side of the tube.
   a) The isolation buffer is kept warm at 37°C in the water bath.

40. Centrifuge cells at 300 × g for 10 min.

41. Aspirate off supernatant, being careful not to disturb pellet.

42. Re-suspend cells by adding 2 mL of the isolation buffer to the cell pellet using a pipette and gently tapping/flicking the tube.

43. Filter cells through 40 μm cell sieve (Falcon, BD Biosciences) into FACS tube.

44. Keep cells on ice and protected from light.

45. Dissociated single cells are sorted using a Cytosebia Influx Cell Sorter (BD Bioscience).
   a) Microglia sorting strategy is as follows: cells are first defined by their forward scatter vs. side scatter, removing small cellular debris. Single cells are highlighted, and tdTomato-positive cells isolated (Figure 8).
   b) For RNA extraction, cells are directly isolated into 300 μL of cell lysis buffer from the Zymo Quick RNA kit in a 1.5 mL RNase-/DNase-free Eppendorf tube. Care should be made to keep the Zymo cell lysis buffer at 22°C–24°C, as the lysis buffer freezes on ice. RNA extraction is performed exactly as according to manufacturer’s instructions (Zymo Research, https://files.zymoresearch.com/protocols/_r1054_r1055_quick-rna_miniprep_kit.pdf), completed immediately after cell isolation.

Figure 8. Representative FACS gating and tdTomato-positive microglia sorting strategy from an uninjured tamoxifen-treated CX3CR1creERT2 × iDTR × tdTomatoFlox mouse with resident microglia
c) From our experience, we have pooled two ipsilateral hippocampi (i.e., the ipsilateral hippocampi collected from two mice) and we are able to isolate ~40k cells from naive mice, ~20–34k resident microglia cells at 3 days post injury, and ~5–15k repopulating microglia cells at 3 days post injury in a mouse unilateral controlled cortical injury model of moderate traumatic brain injury, as previously described in Willis et al., (2020). We have not attempted to isolate the surviving microglia immediately after DT-induced depletion, when the microglia are >90% depleted. Biological replicates can be pooled during FACS isolation prior RNA extraction to generate more RNA, if needed for downstream applications. Troubleshooting 2

⚠ CRITICAL: For all steps, ensure to perform in RNase and DNase-free manner, avoiding any fixatives, as this will affect sample quality. Process no more than 2 samples at a time to ensure that all processing is done as quickly as possible. All steps with papain should be done quickly, and papain incubation timed to ensure tissue is appropriately digested.

EXPECTED OUTCOMES

Microglial depletion/repopulation can be induced using the above protocol. Under naive conditions, microglia will be depleted (>90%) and will undergo endogenous repopulation over several days after DT cessation (Willis et al., 2020). We have noted with microglial repopulation is more rapid under injury conditions, returning to similar levels as sham-operated vehicle-treated mice after three days; see Willis et al. (2020). We recommend checking the depletion efficiency via immunohistochemistry assays, staining and quantifying numbers of microglia (e.g., Iba1pos/Tmem119pos cells) in the region of interest. Representative confocal images and microglial quantification using this protocol can be found within Figure 9 and also Willis et al., 2020. We also recommend that the RNA quality of FACS isolated cells should be checked to ensure it is of sufficient quality and quantity for downstream applications of interest; this can be achieved with a Bioanalyzer (e.g., an Agilent 2100 Bioanalyzer). The required quality and quantity of RNA is dependent on the downstream application/s of interest. Using the above protocol, we have isolated 27–100 ng of total RNA from 25,000 to 67,000 cells (Willis et al., 2020).

![Figure 9. Depletion of microglia in CX3CR1<sup>creERT2</sup> × iDTR × tdTomato<sup>lox</sup> mice](#)

(A) Experimental timeline for tamoxifen treatment followed by saline vehicle or DT administration via IP injection for microglia depletion in CX3CR1<sup>creERT2</sup> × iDTR × tdTomato<sup>lox</sup> mice.

(B) tdTomato<sup>pos</sup> microglia numbers in the dentate gyrus of saline (vehicle) versus DT-treated CX3CR1<sup>creERT2</sup> × iDTR × tdTomato<sup>lox</sup> mice (t = 17.10, df = 8, p < 0.0001, >90% depletion; unpaired Student’s t test). Data are represented as means ± SEM.

(C) Representative confocal images of tdTomato<sup>pos</sup> microglia in CX3CR1<sup>creERT2</sup> × iDTR × tdTomato<sup>lox</sup> mice. Scale bar, 400 μm.
LIMITATIONS
The main limitation of this protocol is the requirement of the transgenic mice (CX3CR1<sup>creERT2</sup> × iDTR mice or CX3CR1<sup>creERT2</sup> × iDTR × tdTomato<sup>flox</sup> mice). However, these mice can be sourced from commercial colonies that are available from The Jackson Laboratory. The timing dependent nature of this pharmaco-genetic model, where a 4–6 week rest period is necessary between tamoxifen treatment and subsequent DT administration, allowing for the turnover of blood-derived monocytes (Parkhurst et al. 2013), may present a limitation for studies that use mice that are less than 9-weeks-old. It is also worth noting that we have observed that systemic DT administration into CX3CR1<sup>creERT2</sup> × iDTR mice induces ~30% depletion of CX3CR1<sup>pos</sup>/tdTomato<sup>pos</sup> macrophages in the choroid plexus, but no change in Iba1<sup>pos</sup> macrophages in the meninges of tamoxifen-treated CX3CR1<sup>creERT2</sup> × iDTR × tdTomato<sup>flox</sup> mice (Willis et al. 2020). Effects on brain macrophage populations (e.g., choroid plexus, meninges) may be avoided through stereotaxic delivery of DT into the region of interest, as we have previously shown that intra-hippocampal DT delivery into tamoxifen-treated CX3CR1<sup>creERT2</sup> × iDTR has no effect on Iba1<sup>pos</sup> macrophage numbers in either the choroid plexus or meninges (Willis et al. 2020).

TROUBLESHOOTING

Problem 1
What if the microglia depletion did not work? (steps 1–3 or step 24)

Potential solution
We recommend checking the DT. If DT is left at room temperature, or the working solution is not used very shortly after being prepared, then it will lose its depletion effectiveness. Avoid any freeze/thaw cycles for DT stock aliquots. Microglial depletion and repopulation using this protocol is shown in representative confocal images in Willis et al., (2020), demonstrated by visualization and stereological quantification of tdTomato-positive or Iba1-positive or Tmem119-positive microglia in CX3CR1<sup>creERT2</sup> × iDTR × tdTomato<sup>flox</sup> mice or CX3CR1<sup>creERT2</sup> × iDTR mice.

Problem 2
What if the microglia yield able to be FACS isolated is very low? (steps 30–45)

Potential solution
Preform all steps quickly and on ice. Cells should be handled very gently when preparing the single cell suspension. Be sure to aspirate against the side of the tube, rather than the bottom of the tube. Care should be paid to the papain solution. Use fresh papain solution; the papain should readily dissociate the tissue within the specified time. If the tissue does not easily dissociate, then the papain may need to be replaced with new stock with the correct concentration.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jana Vukovic (j.vukovic@uq.edu.au).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate/analyze datasets/code.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100211.
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DECLARATION OF INTERESTS
The authors declare no competing interests.

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