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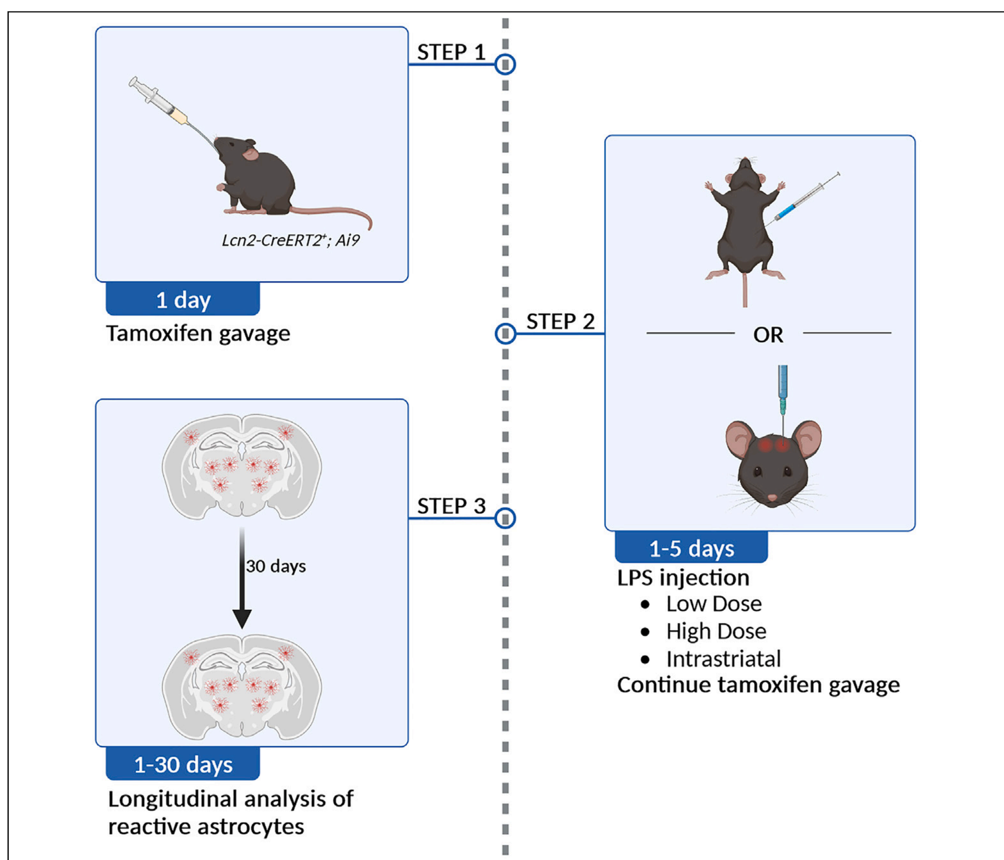
Protocol

Protocol for the longitudinal study of neuroinflammation and reactive astrocytes in *Lcn2CreERT2* mice



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Highlights

Protocol induces neuroinflammation to study reactive astrocytes in *Lcn2CreERT2* mice

Three LPS treatments are described: low-dose, high-dose, and intrastriatal injection

Fluorescent labeling allows longitudinal tracking of reactive astrocytes for weeks

Steps for tissue preparation, sectioning, and immunohistochemistry are provided

During brain disease, astrocytes can reprogram into a reactive state that alters many of their functions. Here, we present a protocol for studying neuroinflammation and reactive astrogliosis in mice using lipopolysaccharide (LPS) from *E. coli*. We describe steps for employing the *Lcn2CreERT2* mouse crossed into a fluorescent Cre reporter line to label a subset of reactive astrocytes during and after inflammation. We then detail procedures for the longitudinal study of reactive astrocytes during the induction, progression, and/or resolution of astrogliosis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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SUMMARY

During brain disease, astrocytes can reprogram into a reactive state that alters many of their functions. Here, we present a protocol for studying neuroinflammation and reactive astrogliosis in mice using lipopolysaccharide (LPS) from *E. coli*. We describe steps for employing the *Lcn2CreERT2* mouse crossed into a fluorescent Cre reporter line to label a subset of reactive astrocytes during and after inflammation. We then detail procedures for the longitudinal study of reactive astrocytes during the induction, progression, and/or resolution of astrogliosis.
For complete details on the use and execution of this protocol, please refer to Agnew-Svoboda et al.[1]

BEFORE YOU BEGIN

1. Identify your institution's animal use regulatory committee and confirm that all experiments conform to their regulatory guidelines and standards.
2. The use of anti-inflammatories as analgesics is not recommended for these procedures since this will attenuate the inflammatory response. The authors suggest working with your institution's regulatory committee and a veterinarian to determine an alternative.

This protocol describes three methods for the induction and labeling of reactive cells in models of neuroinflammation in the *Lcn2CreERT2* mouse. *Lcn2* is used as a driver for CreERT2 expression in this line because it is strongly upregulated in reactive astrocytes in mouse models of neuroinflammation.[1] *Lcn2CreERT2* mice are first crossed into the *Ai9* Cre reporter line and the *Lcn2CreERT2*$^{+}$;*Ai9* offspring are aged to a minimum of 6 weeks prior to beginning one of the three neuroinflammatory induction methods. The low-dose and high-dose LPS protocols are designed to induce systemic inflammation and can be used to broadly study neuroinflammatory-related processes.[2] The injection of LPS into the striatum causes neuroinflammation that leads to a progressive loss of dopaminergic neurons in the substantia nigra.[3] This progressive loss of dopaminergic neurons mirrors the progressive loss seen in Parkinsonian disorders (PD) and can be used to study the role of neuroinflammation in the progression of PD. The role of reactive astrocytes (RAs) in disease onset and progression has been difficult to study. The introduction of new tools to specifically label and target reactive

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astrocytes like the *Lcn2CreERT2* mouse will aid in understanding astrocyte reactivity and the roles of RAs in disease-related processes.

Institutional permissions

All animal procedures detailed in the following sections were performed according to the University of California Riverside's Institutional Animal Care and Use Committee (IACUC) guidelines.

Generate *Lcn2CreERT2;Ai9* mice

Timing: 19 weeks

Before experiments can begin *Lcn2CreERT2$^+$;Ai9* animals must be generated. The lines used in this protocol are *Lcn2CreERT2* and the Cre-dependent reporter line *Ai9*. Both lines are on the C57BL/6 background. There are reports that certain strains may be resistant to LPS challenge.[4] Results may vary if a different strain background is used. The *Ai9* line contains a CAG promoter and a floxed stop sequence followed by a tdTomato sequence inserted at the Rosa locus. Other Cre-dependent reporter lines may be used instead. For example, several Cre-dependent lines driving a variety of genetic tools have been generated by the Allen Institute for Brain Science.[5–8] Of note, we find that the *Ai9* reporter provides robust labeling in astrocytes and intensity of the fluorescent labeling may vary if another reporter is used.

3. Cross *Lcn2CreERT2$^+$* mice with homozygous *Ai9* mice (Jax strain 007909) to obtain *Lcn2CreERT2$^+$;Ai9* mice.
4. Extract genomic DNA from tissue samples and genotype the offspring via PCR for the *Lcn2CreERT2* transgene using the following primers: Forward 5′-GGCAGTCCAGATCTGAG CTGC-3′ and Reverse 5′-TGCATCGACCGGTAATGCAGG-3′.
   a. The expected product size for the transgene is 408 bp.
   b. The provided cycling conditions have been optimized with Promega GoTaq green master mix (cat# M7123). If a different master mix is used the optimal conditions may vary.

Note: A Bio-Rad C1000 Touch thermal cycler is used in this protocol, however any thermal cycler capable of cycling through the required temperatures for the required times would be adequate.

PCR reaction mix

Reagent	Amount
DNA template	1 μL
GoTaq (2×)	5 μL
Forward primer (10 mM)	0.2 μL
Reverse primer (10 mM)	0.2 μL
ddH_2O	3.6 μL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	30 s	39 cycles
Annealing	59°C	30 s	
Extension	72°C	45 min	
Final extension	72°C	5 min	1
Hold	4°C	forever	

5. Age animals to 6 weeks.

⚠ CRITICAL: The authors have not observed reporter induction in the absence of tamoxifen; however, we still suggest the following controls be used: tamoxifen + saline; oil + LPS; and oil + saline. Be sure to generate enough animals for controls.

Tamoxifen

⏲ Timing: 1–24 h

The *Lcn2CreERT2* line uses a tamoxifen-dependent Cre. Thus, the *Lcn2CreERT2*$^{+}$*;Ai9* mice require tamoxifen for recombination and deletion of the stop cassette to allow expression of the red fluorescent protein tdTomato. Frequency and dosage of tamoxifen was determined based on previous studies showing the half-life of tamoxifen metabolites and their biological activity in similar Cre lines.[9,10] The timing of gavage was designed to ensure tamoxifen would be present for Cre to be active during neuroinflammation.[1,3,4,10]

6. Weigh appropriate amount of tamoxifen and add to corn oil to create a stock of 20 mg/mL tamoxifen in corn oil.
7. Protect container from light and place on a shaker for 1–24 h at 37°C until fully dissolved.

Note: See troubleshooting 1.

8. Once tamoxifen has fully dissolved, store at 4°C and protect from light.
   a. Do not use prepared tamoxifen after 1 month from preparation date. It is recommended to make fresh tamoxifen aliquots weekly.
   b. Inspect mixture before each use. It should be clear and free of contaminants.

⚠ CRITICAL: Tamoxifen is toxic; be sure to use all proper precautions and dispose of contaminated waste appropriately.

⚠ CRITICAL: Be sure the corn oil being used is not rancid. Rancid corn oil can affect the survival of the animals. Corn oil should be kept between 20°C and 25°C.

⚠ CRITICAL: While gavage is a simple technique, care must be taken to avoid esophageal damage.

LPS

⏲ Timing: 15 min

Lipopolysaccharides (LPS) from *E. coli* are known to induce an inflammatory response when given intraperitoneally and when injected directly into the brain.[1,3,4]

9. Reconstitute LPS from *E. coli* in sterile saline. The solution will be hazy and may have a yellow tint.
   a. 3.5 mg/mL for low-dose intraperitoneal (IP) injection.
   b. 7.5 mg/mL for high-dose IP injection or intrastriatal injection.
10. Aliquot and store at −20°C.
   a. Avoid freeze-thaw cycles and do not reuse thawed aliquots.
   b. Aliquot size will depend on the number of animals you plan to inject at once. Just prior to low-dose or high-dose injection, LPS will be diluted in sterile saline. Plan your aliquots accordingly as thawed aliquots cannot be refrozen and used later.

c. For low-dose LPS, 25 μL aliquots are typically enough for two 25 g animals. For high-dose LPS, 80 μL aliquots are typically enough for two 25 g animals. The aliquot sizes given are based on usage with low dead space syringes and needles. Do not forget to account for reagent loss in the dead space of syringes and needles when determining aliquot size.
d. The stability of LPS is affected by each freeze-thaw cycle and can be stored at −20°C for up to two years.

⚠ CRITICAL: LPS is extremely toxic to humans. Be sure to use all proper precautions and dispose of contaminated waste properly.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat anti-GFAP (1:500)	Invitrogen	13-0300
Tomato lectin (1:75)	Vector Labs	B-1175-1
Rabbit anti-DsRed (1:500)	Takara	632496
Rabbit anti-Iba1 (1:500)	Fujifilm	019-19741
Chicken anti-Fox3 (NeuN) (1:500)	Aves Labs	NUN-0020
Mouse anti-tyrosine hydroxylase (1:500)	R&D Systems	MAB7566
Chicken anti-mCherry (1:500)	Aves Labs	MCHERRY-0100
Chemicals, peptides, and recombinant proteins		
Lipopolysaccharides from *Escherichia coli*	Sigma-Aldrich	L2880
Sterile saline solution	Covetrus	069169
Tamoxifen	Sigma-Aldrich	T5648
Corn oil	Sigma-Aldrich	C8267
GoTaq Green Master Mix	Promega	M7123
Tissue-Tek O.C.T. compound	Sakura	4583
Fluoro-Gel	Electron Microscopy Sciences	17983
Nail polish	Wet n Wild	Black creme
Sodium chloride	Fisher	BP358-12
Potassium chloride	Fisher	BP366-500
Sodium phosphate dibasic	Sigma	SLBV4963
Potassium phosphate monobasic	Fisher	BP363-500
Triton X-100	Fisher	AAA16046AE
DAPI	Invitrogen	62248
Goat serum	Sigma	G6767
Donkey serum	Sigma	SIAL-S30
Critical commercial assays		
RNAscope Multiplex Fluorescent Detection Kit v2	Advanced Cell Diagnostics	323110
Experimental models: Organisms/strains		
Mouse: Lcn2CreERT2$^+$ (6–24 weeks, male or female)	Riccomagno Lab	N/A
Mouse: Ai9 (6–24 weeks, male or female)	The Jackson Laboratory	Strain #007909
Oligonucleotides		
Lcn2CreERT2 forward primer	IDT	GGCAGTCCAGATCTGAGCTGC
Lcn2CreERT2 reverse primer	IDT	TGCATCGACCGGTAATGCAGG
Other		
Animal feeding needle (gavage needle), 20 g, 1.5″	Fisher	01-208-87
0.5 μL Neuros syringe	Hamilton	65457-01
Embedding mold	Fisher	22-19
Small animal stereotaxic instrument	Kopf	Model 963
Thermal cycler	Bio-Rad	C1000 Touch
U-100 Syringes micro fine needle 28G	BD	329461
0.7 mm Burr for micro drill	FST	19007-07

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
K.1070 High-speed rotary micromotor	Foredom	K.1070
Leica DMi8 A Confocal microscope	Leica	DMi8 A
Leica CM1950 cryostat	Leica	CM1950
Fisherbrand Superfrost Plus microscope slides	Fisher	12-550-15
Slide mailer	Fisher	HS15986
Slide box holder	Fisher	03-448-286

MATERIALS AND EQUIPMENT

1× PBS (pH 7.4)

Reagent	Final concentration	Amount
Sodium chloride	137 mM	8 g
Potassium chloride	2.7 mM	0.2 g
Sodium Phosphate Dibasic	10 mM	1.44 g
Potassium Phosphate Monobasic	1.8 mM	0.245 g
Distilled water	N/A	Fill to 1 L
Adjust pH to 7.4	N/A	N/A
Total	**N/A**	**1 L**

Store at 20°C–25°C. May be stored indefinitely. Check for contaminants before each use.

Blocking solution

Reagent	Final concentration	Amount
Serum (goat or donkey)	10%	500 mL
Triton X-100	0.1–0.3%	5–15 mL
1× PBS	N/A	fill to 5 mL
Total	**N/A**	**5 mL**

Store at 4°C for up to 1 week. Check for contaminants before each use.

Antibody solution

Reagent	Final concentration	Amount
Serum (goat or donkey)	1%	50 mL
Triton X-100	0.1%	5 mL
1× PBS	N/A	fill to 5 mL
Total	**N/A**	**5 mL**

Store at 4°C for up to 1 week. Check for contaminants before each use.

STEP-BY-STEP METHOD DETAILS

Low-dose LPS

Timing: 8–30 days

In *Lcn2CreERT2;Ai9* mice, low-dose IP LPS treatment will induce systemic inflammation, including reactive astrogliosis. When given tamoxifen during this inflammation the result will be fluorescent labeling of a subset of reactive cells.[1] Tissue may be harvested on day 8 or 30 for longitudinal studies of labeled astrocytes. A summary of the timing for low-dose treatments can be found in Figure 1. Expected outcomes can be found in Figure 4.

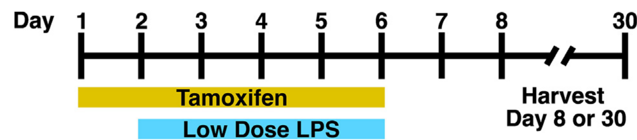

Figure 1. Summary of the timing of treatments for low-dose LPS protocol

1. On day 1, weigh each animal and calculate the amount of tamoxifen in corn oil (20 mg/mL) to orally gavage at 150 mg/kg.
   a. Warm tamoxifen in corn oil to 20°C–25°C for at least 15 min prior to oral gavage.
2. Using an animal feeding needle (Fisher), orally gavage the appropriate amount of tamoxifen in corn oil for each animal.

   Note: See troubleshooting 2.

3. On day 2, weigh each animal and orally gavage tamoxifen as done on day 1.
4. Just prior to LPS injection, add sterile saline to a fresh LPS aliquot thawed on ice to a final concentration of 0.35 mg/mL.
   a. Mix well and briefly vortex if needed. Store on ice until injection.
5. Calculate amount of LPS to be injected so that each animal receives 1.5 mg/kg.
   a. It is not recommended to inject a volume greater than 10 mL per kg of body weight.
6. Hand-restrain the animal and inject appropriate amount of LPS intraperitoneally.

   Optional: Use of syringes and needles with low dead space such as insulin syringes is preferred to avoid loss of LPS but is not required.

   Note: See troubleshooting 3.

7. Repeat steps 3–6 for days 3–6.
8. Harvest tissue on day 8 or any desired time after gavage if performing longitudinal studies. We have harvested brains up to 30 days after gavage.

High-dose LPS

Timing: 4–30 days

High-dose LPS treatment will induce systemic inflammation, including reactive astrogliosis.[10] Treating *Lcn2CreERT2;Ai9* mice with a single high-dose of LPS will result in the fluorescent labeling of reactive cells, including astrocytes. Tissue may be harvested as soon as day 4 or may be harvested at day 30 for longitudinal studies of labeled astrocytes. A summary of the timing for high-dose treatments can be found in Figure 2. Expected outcomes can be found in Figure 5.

9. On day 1, weigh each animal and calculate amount of tamoxifen in corn oil (20 mg/mL) to orally gavage at 150 mg/kg.
   a. Warm tamoxifen in corn oil to 20°C–25°C for at least 15 min prior to oral gavage.
10. Orally gavage the appropriate amount of tamoxifen in corn oil for each animal.

    Note: See troubleshooting 2.

11. On day 2, weigh each animal and orally gavage tamoxifen as done on day 1.
12. Just prior to LPS injection, add sterile saline to a freshly thawed LPS aliquot to a final concentration of 3 mg/mL.
    a. Mix well and briefly vortex if needed. Store on ice until injection.

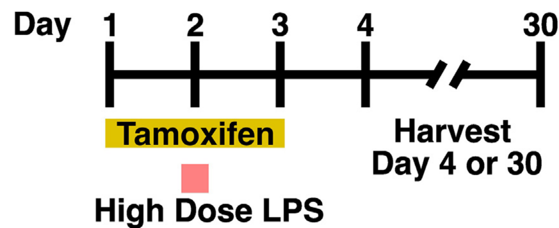

Figure 2. Summary of the timing of and treatments for high-dose LPS protocol

13. Calculate the amount of LPS to be injected so that each animal receives 10 mg/kg.
14. Appropriately restrain the animal and inject LPS intraperitoneally.

    Optional: Use of syringes and needles with low dead space such as insulin syringes are preferred to avoid loss of LPS but are not required.

    Note: See troubleshooting 3.

15. Orally gavage appropriate amount of tamoxifen in corn oil for each animal.

    Note: See troubleshooting 2.

16. On day 3, orally gavage with tamoxifen in corn oil.

    Note: See troubleshooting 2.

17. Harvest tissue on day 4 or any desired time after gavage if performing longitudinal studies. We have harvested brains up to a month after gavage.

Intrastriatal LPS

Timing: 9 days

In *Lcn2CreERT2*$^{+}$*;Ai9* mice, intrastriatally-injected LPS will induce Parkinson-like symptoms and will result in fluorescent labeling of reactive cells including reactive astrocytes.[1,3] Tissue may be collected as soon as day 9 or later for longitudinal studies of labeled astrocytes. A summary of the timing of intrastriatal treatments can be found in Figure 3. Expected outcomes can be found in Figure 6.

18. On day 1, administer tamoxifen in corn oil via oral gavage at a dose of 150 mg/kg.

    Note: See troubleshooting 2.

19. On day 2, administer tamoxifen as done on day 1.

    Note: See troubleshooting 2.

20. Thaw one 7.5 mg/mL aliquot of LPS just prior to LPS injection.
    a. Mix well and vortex briefly if needed. Store on ice until injection.
21. Anesthetize animal with Isoflurane in an induction chamber before mounting on a stereotaxic rig.
22. Shave and aseptically prepare the incision site once the animal is fully anesthetized.

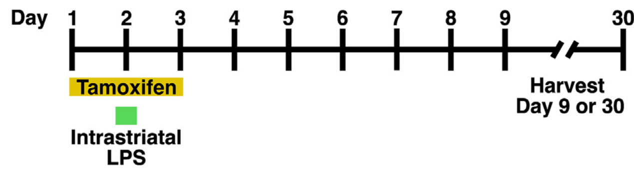

Figure 3. Summary of the timing of treatments for intrastriatal LPS protocol

   a. Sufficient anesthesia is determined by the relaxed posture of the animal and insensitivity to toe and tail pinches.
   b. The authors find that maintaining Isoflurane at 1.5–2% is sufficient to keep the animal anesthetized at the appropriate level throughout the procedure.

23. Make an incision along the midline on the top of the head to expose the skull.
24. Level bregma and lambda landmarks on the skull.
25. Drill holes in the skull using a 0.7 mm burr at the four following coordinates from Bregma: A/P +1.18 mm, M/L ±1.5 mm and A/P −0.34 mm, M/L ±2.5 mm.
26. Load 1.5 μL of 7.5 mg/mL LPS into a Hamilton Neuros syringe.
27. Inject 250 nL of 7.5 mg/mL LPS into each of four locations at a rate of 0.5 μL/min, for a total of 1 μL LPS injected. After each injection leave needle in place for 5 min prior to withdrawing.
    a. Coordinates for injection from bregma: A/P +1.18 mm, M/L ±1.5 mm, D/V −3.5 mm and A/P −0.34 mm, M/L ±2.5 mm, D/V −3.2 mm.

Note: See troubleshooting 3.

28. After the last injection, close skin with Michel Suture Clips.

Optional: Michel Suture Clips are used in this protocol; however alternate suture clips or other wound closure methods may be used.

29. Give 200 μL of sterile saline subcutaneously.
30. Monitor the animal after surgery on a heating pad until awake and freely moving.

⚠ CRITICAL: Heating pad must not be warmer than 37°C. Warmer temperatures may burn and injure the animal.

31. On day 3, administer tamoxifen as on day 1.

Note: See troubleshooting 2.

32. Harvest tissue on day 9 (7 days after LPS injection) or later for longitudinal studies.
    a. If doing longitudinal studies, suture clips must be removed 10–14 days after surgery.

Cryopreservation and sectioning of tissue

⏲ Timing: 2–3 days

Cryopreservation of tissue is performed prior to sectioning on a cryostat. Preparation and sectioning of cryopreserved tissue is performed as follows.

33. After perfusion and tissue collection, brains are fixed in 4% PFA for 12–16 h at 4°C in a 6-well plate filled with approximately 8 mL.
34. Rinse tissue 3× in PBS, filling the well each time (~8 mL).
35. Move brains to 30% sucrose in 1× PBS and store at 4°C for 12–16 h.

36. The next day replace 30% sucrose in 1×PBS. If the brain floats, incubate for 12–16 h again at 4°C. Repeat sucrose replacement as necessary until brain sinks.
37. Air dry brains on a paper towel for 5–10 min.
38. Rinse brains in OCT to coat and remove any surrounding bubbles.
39. Put the OCT-coated brain into an embedding mold filled with OCT.

Note: We recommend notating the orientation of the brain on the outside of the embedding mold before freezing. The orientation of the brain will not be visible after freezing since OCT becomes opaque once frozen.

40. Place mold on dry ice until fully frozen and store at −80°C until ready to cut on a cryostat.

Pause point: Tissue may be stored for up to 1 year at −80°C.

41. Cut 15–20 μm sections on a cryostat and mount onto charged slides.
    a. Cut 15 μm sections if preparing for RNAscope.

Note: See troubleshooting 4.

42. After cutting, let the tissue dry on the slide for 2 h.

Note: Cryosections can be used for RNAscope and/or immunohistochemistry (IHC). If doing RNAscope follow the manufacturer's suggested instructions. If doing IHC, proceed to the immunohistochemistry section of this protocol. Alternatively, slides can be stored at −80°C for future use but the drying step must be performed prior to storage.

a. If you plan on performing a combination of RNAscope and IHC, complete the RNAscope procedures first and begin the immunohistochemistry procedures beginning at step 46.

Pause point: Cut tissue may be stored in a slide box at −80°C for up to 1 year provided they are protected from moisture and frost. This can be done by wrapping the slide box in parafilm and/or storing them with a desiccant. If RNAscope will be performed on the tissue it is not advised to store sectioned tissue for longer than 3 months.

Immunohistochemistry

Timing: 2 days

Immunohistochemistry is performed as follows.

43. Place slides into a slide mailer filled with PBS and incubate at 20°C–25°C for 15 min.
    a. If OCT is not removed after 15 min, repeat PBS wash.

⚠ CRITICAL: Treat slides gently. Vigorously pouring PBS or other liquids directly onto slides may result in the tissue dislodging.

⚠ CRITICAL: Tissue should not be allowed to dry at any time beyond this point. Allowing the tissue to dry will drastically reduce the quality of your tissue and IHC.

44. Dry the slide around the tissue gently while taking care to keep the tissue moist.
45. Draw around the tissue with a hydrophobic pen and let dry while keeping the tissue moist.

a. This may require gently pipetting PBS directly on top of your tissue occasionally while the hydrophobic barrier dries.

46. Block sections with blocking solution for 1 h at 20°C–25°C in a humidifying chamber with enough volume to cover tissue.

Note: It is helpful to estimate the amount of solution required to cover your tissue at this step. This estimation can be used to prepare primary and secondary antibodies in future steps. For example, if 1 mL of blocking solution is needed, then you may make only 1 mL of primary or secondary antibody mix.

Note: A simple humidifying chamber can be made from a plastic slide box holder with damp paper towels at the bottom.

Note: Serum type will depend on your antibodies. Be sure to match the serum type to the secondary antibody host. If using goat secondary antibodies, goat serum is preferred.

a. If performing IHC after RNAscope, block with 3% bovine serum albumin (BSA) in 1× PBS.

47. Mix primary antibodies in antibody solution and store at 4°C or on ice until use.

Note: This step can be done during the 1 h blocking step to save time.

48. Remove blocking solution from slides and place them back into the humidifying chamber.
49. Gently pipette primary antibody mix onto sections and incubate for 12–16 h at 4°C in a humidifying chamber. Use enough volume to sufficiently cover the tissue.
    a. If using tomato lectin, add in with primary antibodies.
    b. If performing IHC after RNAscope, mix antibodies and/or lectin in 3% BSA in PBS.
50. Wash slides in a slide mailer filled with PBS for 10 min 3× at 20°C–25°C.
51. Mix secondary antibodies in antibody solution and store at 4°C or on ice until use.

Note: This step can be done during the PBS washes to save time.

52. Place slides back into the humidifying chamber.
53. Gently pipette secondary antibody mix onto sections and incubate slides at 20°C–25°C in a humidifying chamber for 1–2 h. Use enough volume to sufficiently cover the tissue.
    a. DAPI at 1:1000 dilution may also be added at this step if desired to label cell nuclei.
    b. If using tomato lectin, a fluorescently-conjugated streptavidin is added at this step.
    c. If performing IHC after RNAscope, mix secondary antibodies and/or streptavidin in 3% BSA in PBS.
54. Wash slides in a slide mailer filled with PBS for 10 min 3× at 20°C–25°C.
55. Mount with Fluoro-Gel (or other desired mounting medium) and coverslip, let dry.
56. Paint nail polish around the edges to seal the coverslip.
57. Store at 4°C until ready to image.

Note: See troubleshooting 5.

⚠ CRITICAL: Image slides within one month of completion of IHC or RNAscope.

EXPECTED OUTCOMES

Low-dose, high-dose, and intrastriatal injections of LPS, when combined with tamoxifen treatments, result in tdTomato-positive reactive cells (Figures 3, 4, and 5).[1] In the thalamus, low-dose LPS and tamoxifen treatment result in 388 ± 84 labeled cells per 20 μm section one day after the last treatment (Figure 4). Similar numbers are observed 1 month after treatment (Figure 4).[1] Of the

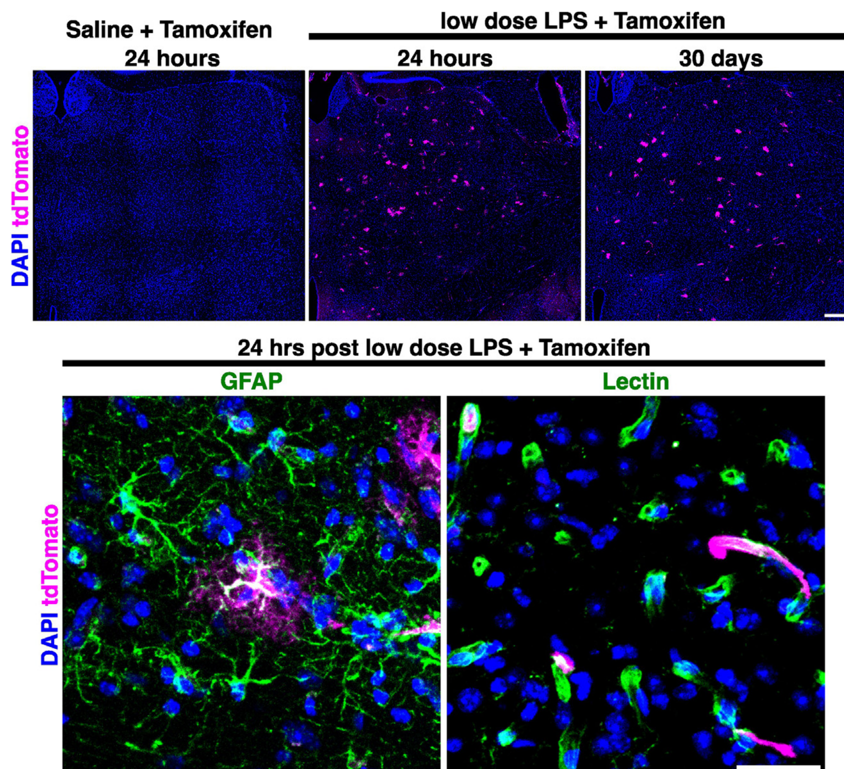

Figure 4. Low-dose LPS expected outcome

tdTomato fluorescent reporter labeling of primarily reactive astrocytes and endothelial cells within the thalamus. Scale 300 μm top, 30 μm bottom. Sections are 20 μm in thickness. Images were taken on a Leica DMi8 M confocal microscope.

tdTomato-labeled cells, 45.2 ± 1.8% are co-positive for the astrocytic marker GFAP, and 50.5 ± 2.2% are co-positive for lectin (Figure 4). The morphology of the lectin-positive cells suggests they are endothelial cells. Notably, no tdTomato-positive cells are co-positive for the neuronal marker NeuN, and only a small percentage (1% ± 0.4%) are co-positive for the microglial marker Iba1. Similar results are observed with high-dose LPS and tamoxifen treatment (Figure 5).[1] Seven days after intrastriatal injection of LPS, approximately 715 ± 164 and 1010 ± 87 tdTomato-positive cells per 20 μm section are observed in the striatum and thalamus, respectively (Figure 6).[1]

LIMITATIONS

These protocols were performed with animals on the C57BL/6N background. There are reports that LPS injection in certain strains may be resistant to LPS challenge. Keep this in mind when crossing *Lcn2CreERT2* animals onto different strain backgrounds.[4]

The second potential limitation of using these approaches is that *Lcn2CreERT2* is also expressed in some non-astrocytic cells (mainly endothelial cells) following LPS injection.[10] While this makes the *Lcn2CreERT2* line a potentially useful tool for the study of reactive endothelial cells in brain injury and disease, to selectively label reactive astrocytes we recommend using a combinatorial approach or co-labeling with an astrocyte-specific marker.

Finally, Lcn2$^+$ astrocytes represent only a subset of reactive astrocytes and there seems to be regional specificity to the expression of *Lcn2CreERT2* in these cells.[1,11–13] This should be carefully considered when interpreting observations stemming from the use of this system.

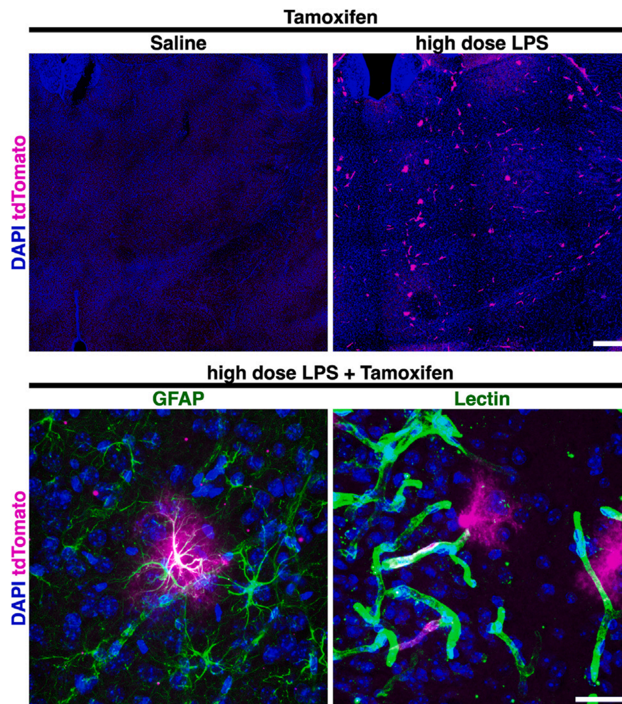

Figure 5. High-dose LPS expected outcome
tdTomato fluorescent reporter labeling of primarily reactive astrocytes and endothelial cells. Scale 300 μm top, 30 μm bottom. Sections are 20 μm in thickness. Images were taken on a Leica DMi8 M confocal microscope.

TROUBLESHOOTING

Problem 1

Tamoxifen crystals do not dissolve in corn oil.

Potential solution

- Occasionally tamoxifen may not enter solution. Vigorously vortexing or sonicating may help to dissolve any difficult crystals.

Problem 2

Animals are difficult to gavage, are injured during gavage, or perish shortly after oral gavage.

Potential solution

- Inspect the gavage needle before each use and discard if there are any rough or sharp areas. Esophageal tissue is delicate and can be easily damaged by rough edges on the gavage needle.
- It is not recommended to orally gavage more than 10 mL per kg body weight.
- Measure the end of the needle to the caudal portion of the sternum and visually note or mark where the mouth of the animal meets the needle shaft. Do not advance the needle further than this point as internal injury to the animal is likely.
- Ensure the scruff of an animal is secure. A mobile head is dangerous for the animal and the person performing the gavage. A proper scruff ensures that the animal's head is not mobile. Starting the scruff closer to the head and tucking the tail in between the third and little fingers often helps to secure the animal. The authors find that an improper scruff is often the case with new users and is what causes the most difficulty.

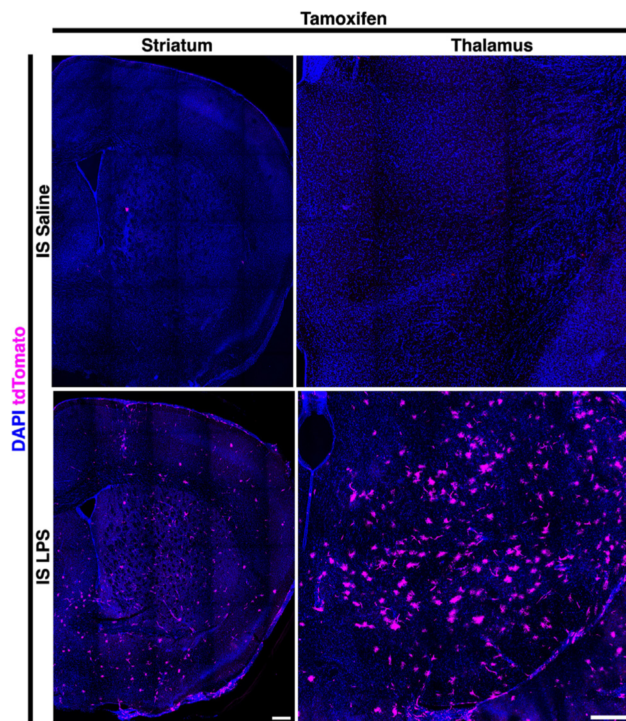

Figure 6. Intrastriatal LPS expected outcome
tdTomato fluorescent reporter labeling of primarily reactive astrocytes and endothelial cells in the striatum and thalamus. Scale 300 μm. Sections are 20 μm in thickness. Images were taken on a Leica DMi8 M confocal microscope.

- Begin gavage needle insertion on the animal's left (your right) side and gently guide it to the back of the throat, then gently guide the needle down. Gavage needle should easily slide down the esophagus, no force should be required to advance the needle. This should be done in one smooth movement. If any force is required to advance the needle, remove it and try again.
- The gavage needles provided in this protocol are straight, have a metal shaft, and a plastic bulb at the tip. This is based on user preference. There are many variations of feeding needles available and users may find they are more comfortable using a different type. It is not recommended that users new to oral gavage use a gavage needle with a plastic shaft as the animal may bite through this.

Problem 3

No or low numbers of reactive astrocytes are labeled after injection.

Potential solution

- Mix LPS thoroughly.
- Check that LPS has not expired or experienced multiple freeze-thaw cycles.

Problem 4

Tissue is cracked, difficult to cut, or otherwise looks poor.

Potential solution

- Freeze tissue shortly after collection. Longer times between collection and freezing will result in more degradation of tissue.
- Thaw tissue to −20°C for 1 h prior to sectioning on cryostat.

- Change to a fresh blade.
- Cut tissue within one year of freezing.
- Ensure tissue does not dry at any point beyond step 43.
- Try a gentler treatment of tissue on slides.

Problem 5

IHC signal intensity is low or poor.

Potential solution

- It is best to image slides within one month of completion of IHC.
- Change post-fix timing. Longer fixation times may mask epitopes. A 2-h or a 4-h fixation time may yield better results.
- Increase Triton X-100 concentration. Nuclear epitopes tend to require more permeabilization.
- Change antibody concentrations.
- Check that antibodies are not old, expired, or have been mishandled.
- All antibodies used in the original paper[1] are known to work well with 12–16 h fixation at a 1:500 dilution or 1:75 dilution for tomato lectin. Some antibodies may work best on vibratome floating sections (not frozen).
- RNAscope is harsh on tissue and not all epitopes will survive this process. Using the weakest protease possible during the RNAscope protocol will increase the chances of an antibody working. Fox3 (Encor, CPCA-FOX3) is one of the antibodies known to our lab to stain poorly after RNAscope. GFAP (Invitrogen, 13-0300) and ALDH1L1 (Cell Signaling, 85828S) work well after the RNAscope process.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Martin Riccomagno (martin.riccomagno@ucr.edu).

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Materials availability

The *Lcn2CreERT2* mouse line is available from the Riccomagno, Fiacco, and Wilson labs upon request.

Data and code availability

This study did not generate and analyze datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.H.W., T.A.F., and M.M.R.; methodology, T.U., W.A.-S., and M.M.R.; investigation, T.U., W.A.-S., and Z.A.F.; visualization, T.U. and M.M.R.; writing – original draft, T.U. and M.M.R.; writing – review and editing, T.U., Z.A.F., E.H.W., T.A.F., and M.M.R.; funding acquisition, E.H.W., T.A.F., and M.M.R.; supervision, E.H.W., T.A.F., and M.M.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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