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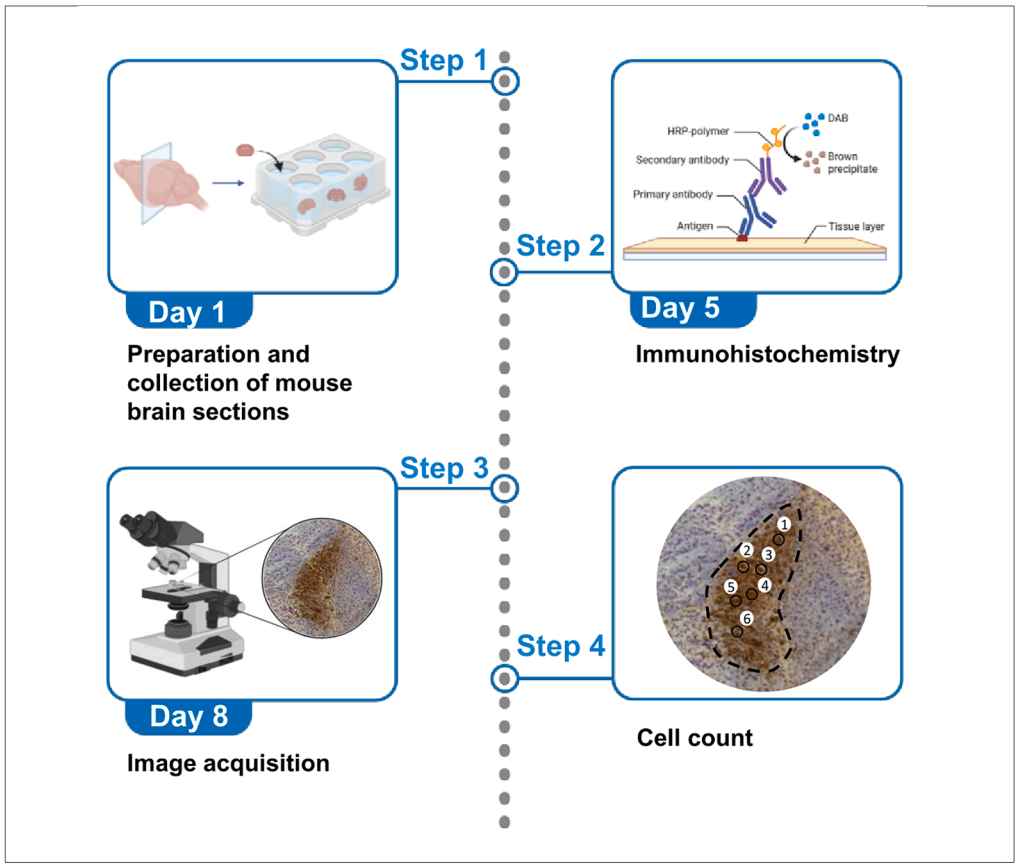
Protocol to count the number of noradrenergic neurons in mouse locus coeruleus by unbiased stereology



Loss of noradrenergic (NE) neurons in the locus coeruleus (LC) occurs in both Alzheimer's disease (AD) and Parkinson's disease (PD): pharmacological therapies aimed at neuroprotection of this brain area are needed. Here, we present a protocol for assessing the absolute number of NE neurons in the mouse LC by unbiased stereological counting. We describe steps for preparing the brain slices, delineating the region of interest, counting the neurons, and using the Stereo Investigator software.

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

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Highlights

Protocol for assessing the absolute number of neurons in the mouse locus coeruleus

Steps for brain preparation, immunostaining, image acquisition, and counting

Instructions for using the Stereo Investigator software

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Protocol to count the number of noradrenergic neurons in mouse locus coeruleus by unbiased stereology

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SUMMARY

Loss of noradrenergic (NE) neurons in the locus coeruleus (LC) occurs in both Alzheimer's disease (AD) and Parkinson's disease (PD): pharmacological therapies aimed at neuroprotection in brain area are needed. Here, we present a protocol for assessing the absolute number of NE neurons in the mouse LC by unbiased stereological counting. We describe steps for preparing the brain slices, delineating the region of interest, counting the neurons, and using the Stereo Investigator software.

For complete details on the use and execution of this protocol, please refer to Regoni et al.[1]

BEFORE YOU BEGIN

This protocol details the specific procedures for unbiased stereological counting of absolute numbers of noradrenergic (NE) neurons in the locus coeruleus (LC) of mice. We have successfully applied this protocol to C57BL6/N mice at 1, 6, and 12 months of age.[1]

Importantly, this protocol is also applicable to other mouse strains and disease models. This makes it a valuable tool for investigating LC degeneration associated with aging or diverse pathological conditions, and for assessing neuroprotective strategies.

Innovation

The LC is a minute brain structure, which presents difficulties in access, sampling, and analysis, particularly in murine models. Standardized quantitative methods for its NE population are currently lacking in the literature. To assess the efficacy of neuroprotective therapies, a precise quantitative method is essential. For the first time, this protocol introduces a systematic workflow for sampling the LC, performing staining procedures, and achieving unbiased quantification of absolute NE neuron numbers via Stereo Investigator software. This protocol represents an important advance in the study of the LC at the preclinical level.

Institutional permissions

All experiments were designed to minimize the number of euthanized animals, in compliance with the 3Rs (replacement, reduction, and refinement) principle. Mice were housed and bred at the

San Raffaele Hospital animal facility, strictly complying with both institutional guidelines and international regulations. These include EU Directive 2010/63/EU, EEC Council Directive 86/609 (OJL 358, 1, December 12, 1987), and the NIH Guide for the Care and Use of Laboratory Animals (U.S. National Research Council, 1996).

All procedures were approved by the Institutional Animal Care and Use Committee from the Italian Ministry of Health, under protocol number DC8BD.399. Researchers planning to use this protocol must obtain authorization and training from their institution.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Anti-Tyrosine Hydroxylase (TH, Tyrosine Monooxygenase) Monoclonal antibody, Unconjugated, Clone 2/40/15	Millipore	Cat# MAB318; RRID: AB_2201528
Sheep Anti-Mouse IgG - Horseradish Peroxidase (HRP)	GE HealthCare	Cat# NA931; RRID: AB_772210
Biological samples		
Mouse brain tissue (C57BL6/N mice, aged 1, 6, 12 months)	IRCCS San Raffaele Institute	N/A
Chemicals, peptides, and recombinant proteins		
Ketamine hydrochloride (Narketan 10)	Vetoquinol	N/A
Xylazine hydrochloride	Sigma-Aldrich	CAS: X1251-1G
Paraformaldehyde (PFA)	Sigma-Aldrich	CAS: 30525-89-4
Sodium chloride (NaCl)	Sigma-Aldrich	CAS: 7647-14-5
Sodium hydroxide (NaOH)	Merck	CAS: 310-73-2
Sucrose	Sigma-Aldrich	CAS: 57-50-1
2-Methylbutane	Sigma-Aldrich	CAS: 78-78-4
Ethylenedinitrilotetraacetic acid disodium salt dihydrate (EDTA)	Merck Millipore	CAS: 6381-92-6
Dulbecco's Phosphate-Buffered Saline (DPBS)	Thermo Fisher Scientific	CAS: 14200-059/-091
Sodium azide (NaN_3)	BDH	CAS: 247-852-1
Hydrogen peroxide solution 30% (H_2O_2)	Sigma	CAS: 7722-84-1
O.C.T compound (Killik - O.C.T.)	Bio-Optica	CAS: 23076
Cresyl violet acetate	Sigma-Aldrich	CAS: 10510-54-0
Triton X-100	Sigma	CAS: 9036-19-5
Bovine Serum Albumin (BSA)	Sigma	CAS: 9048-46-8
Gelatin (food grade)	Giacomini	N/A
Chromic potassium sulfate ($KCr(SO_4)_2$)	BDH	CAS: 7788-99-0
ddH_2O	–	N/A
Glacial acetic acid (CH_3COOH)	Sigma-Aldrich	CAS: 64-19-7
Chloroform solution ($CHCl_3$)	Sigma-Aldrich	CAS: 67-66-3
Ethanol (EtOH)	Merck	CAS: 64-17-5
Xylene	Carlo Erba	CAS: 1330-20-7
Eukitt Quick-hardening mounting medium	Sigma-Aldrich	CAS:25608-33-7
Critical commercial assays		
DAB Substrate Kit	Abcam	Cat# Ab64238
Experimental models: organisms/strains		
C57BL6/N mice, aged 1, 6, 12 months	Charles River	Cat# 027
Software and algorithms		
Stereo Investigator software	MBF Bioscience	RRID: SCR_024705

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Filter paper	Ettore Pasquali	Cat# 5520045
Leica Cryostat 1860	Leica	RRID:SCR_025772
12-Multiwell plate	Corning Incorporated	Cat# CLS3513
Coverglass for automated coverslippers	Leica Biosystems	Cat# 14016043326
Omnican 50 Type LDS syringe	B. Braun	Cat# PRID00000050
Mayo TC Operating Scissors, 14 cm curved	Medicon	Cat# 03.51.64
Surgical Scissors, Sharp, 12 cm	Fine Science Tools	Cat# 14002-12
Dumont #55 Forceps, 11 cm	Fine Science Tools	Cat# 11255-20
Dumont #7 Forceps, 11 cm	Fine Science Tools	Cat# 11271-30

MATERIALS AND EQUIPMENT

Gelatin-coating solution		
Reagent	Final concentration	Amount
Gelatin	0.5%	1.25 g
Chromium potassium sulfate	0.25%	0.625 g
ddH_2O	-	250 mL
Total	-	**250 mL**

Note: The solution can be stored at 4°C for 48 h but it is recommended to prepare and use it on the same day.

Fixation buffer (4% paraformaldehyde [PFA] buffer)		
Reagent	Final concentration	Amount
Paraformaldehyde [PFA]	4% (1.3M)	8 g
10N NaOH	-	Few drops
10X PBS	1X	20 mL
ddH_2O	-	180 mL
Total	-	**200 mL**

⚠ CRITICAL: Paraformaldehyde is highly toxic. Avoid contact with skin, eyes, or mucous membranes. Avoid breathing the powder. During preparation, always wear a lab coat, mask, and gloves, and work under a fume hood.

Warm the solution to 60°C; ensure it does not boil. Temperatures above 60°C denature paraformaldehyde, making it useless as a fixative.

The powder does not immediately dissolve into solution. Slowly raise the pH by adding 10N NaOH dropwise from a pipette until a clear solution is formed.

Note: The solution can be stored at 4°C for 4 weeks. For long-term storage (up to a year), the solution can be aliquoted and stored at −20°C; thawing on ice 3 h in advance will ensure the solution is at 4°C when needed for use.

Alternatives: Antigenfix (Diapath, P0016) can be used as alternative fixative.

Cryoprotection buffer (30% sucrose solution)		
Reagent	Final concentration	Amount
Sucrose	30% (0.87 M)	30 g
10X PBS	1X	10 mL
ddH_2O	-	90 mL
Total	-	**100 mL**

Note: The solution can be stored at 4°C for a week but it is recommended to prepare and use it on the same day.

Cresyl violet solution		
Reagent	Final concentration	Amount
Cresyl Violet	0.1%	0.1 g
Glacial acetic acid	0.3%	300 µL
ddH_2O	-	100 mL
Total	-	**100 mL**

Note: The solution can be stored at 20°C–22°C in a dark place and it is stable for 6 months.

Blocking solution		
Reagent	Final concentration	Amount
Triton X-100	0.3%	150 µL
BSA	2%	1 g
1X PBS	-	Fill up to 50 mL
Total	-	**50 mL**

Note: The solution can be stored at 4°C for a week. For long-term storage (up to a year), the solution can be aliquoted and stored at −20°C; thaw on ice before use.

Incubation solution for antibody		
Reagent	Final concentration	Amount
Triton X-100	0.3%	150 µL
BSA	1%	0.5 g
1X PBS	-	Fill up to 50 mL
Total	-	**50 mL**

Note: The solution can be stored at 4°C for a week. For long-term storage (up to a year), the solution can be aliquoted and stored at −20°C; thaw it on ice before use.

Note: Triton X-100 solution is viscous and should be pipetted with care to ensure precision in volume measurements.

STEP-BY-STEP METHOD DETAILS

Preparation of gelatin-coated slides

Timing: 1 day

1. Prepare the gelatin-coating solution by dissolving 1.25 g of gelatin in 250 mL of heated, deionized H_2O (temperature should not exceed 37°C).
2. After the gelatin has dissolved, add 0.625 g of chromium potassium sulfate.

Note: Chromium potassium sulfate dodecahydrate will positively charge the slides allowing them to attract negatively charged tissue sections.

3. Cool to 20°C–22°C and filter.

⚠ CRITICAL: Change the filter paper several times to avoid the formation of agglomerates.

4. Place the slides into metal racks.

Note: The slides must be cleaned in advance by dipping them in 100% ethanol for 10 s.

5. Dip the racks containing the slides into the gelatin-coating solution for 1 min.
6. Remove the racks containing the slides and let them drain.

Note: Blot excess solution from the racks onto filter paper (gently tap the racks against the filter paper for better drainage).

7. Place the racks containing the slides on the lab bench and cover them with paper towels to protect them from dust.
8. Dry at 20°C–22°C for 16 h.
9. Dried slides can be stored at 4°C.

Note: The solution can be stored at 4°C for 48 h, but it is recommended to prepare and use it on the same day.

Collection and preparation of the mouse brain

Timing: 2 days

The section below details the perfusion and tissue fixation protocol for harvesting mouse brain.

⚠ CRITICAL: Keep the time between perfusion and euthanasia of the animal and fixation as short as possible, for best preservation of tissue morphology.

Day 1

10. Animal preparation.
    a. Anesthetize the mouse by intraperitoneal injection of a mixture of ketamine/xylazine (100 and 10 mg/kg, respectively).
    b. Place the mouse back to its cage and wait for ~5 min.
    c. Use toe pinch response method to determine depth of anaesthesia.

Note: Animal must be unresponsive before continuing.

11. Transcardiac perfusion.

Note: From this step on, please operate in fume hood.

a. Place the mouse on a styrofoam plate with abdomen facing up. Firmly pin down its four paws to the surface extending them as wide as possible using small needles.
b. Grip the skin on the chest with forceps and make an incision through the abdominal wall just beneath the rib cage to expose the diaphragm and the liver.
c. Continue the diaphragm incision upward along the entire length of the rib cage.
d. Cut vertically along the entire length of the sternum; open both sides of the rib cage and pin them to the surgical station (or cut them off) to fully expose the heart and lungs.
e. Secure the heart with dissecting forceps at a steady position.
f. Insert a 25-gauge needle (connected to the tube of a peristaltic pump) into the posterior end of the left ventricle.

⚠ CRITICAL: During the insertion of the needle into the left ventricle, it is critical that the needle does not advance too much into the right ventricle or even pierce through the heart.

g. Turn pump on to let saline flow. The heart will start to swell. Cut the right atrium using fine scissors. Dark venous blood should flow out immediately.
h. Perfuse with 25 mL ice-cold phosphate buffer saline (PBS) solution at 0.25 mL/s flow rate.
i. As saline flushes out the blood, the liver gradually turns pale.

⚠ CRITICAL: In order for saline solution to flow properly into the brain, the heart should be beating at the beginning of this process. It is crucial to watch the time between anaesthesia injection and perfusion (max 5 min).

j. Stop the pump and switch from saline to Fixation Buffer; make sure no air bubble gets into the perfusion system during the switch.
k. Perfuse with 25 mL ice-cold Fixation Buffer at the same flow rate as PBS.

⚠ CRITICAL: As fixative goes into circulation, body twitching, tail flicking and head moving can be observed: when these phenomena happen, perfusion is considered to be successful.

l. At the end, remove the needle and unpin the mouse from surgical station. The mouse should be stiff at this stage.

12. Brain dissection.
a. Decapitate the mouse with tissue dissecting scissors by making a cut caudally to the ears.
b. Make an incision along the midline from the neck to the nose and then reflect the two flaps of skin laterally to expose the skull.
c. Hold the head tightly with one hand. Trim off bones at the caudal end with dissecting scissors and clear off any residual muscle on the skull with forceps or scissors.
d. Make a lateral cut into the foramen magnum (underneath the brain) using fine scissors.
    i. Repeat the cut on the contralateral side.
    ii. Carefully remove the skull covering the cerebellum.
e. Starting from the caudal part, cut the cranium along the middle-sagittal suture using fine scissors; carefully remove the skull with forceps to expose the brain.
f. Cut the optic nerves and carefully scoop the brain using a micro spatula.

g. Remove the brain and place it in a 15 ml tube containing 5 ml of Fixation Buffer for post-fixation for 16 h at 4°C.

Day 2

13. Brain snap freezing and cryoprotection.
    a. Following post-fixation, transfer the brain to a 15 ml tube containing 12 mL of 30% sucrose solution in PBS at 4°C for 24–48 h (or until the brain sinks to the bottom).
    b. Dip the brain for 30 s into a beaker containing isopentane placed in dry ice and then store at −80°C.

Preparation of mouse brain sections using cryostat

Timing: 1–2 h

This section outlines the procedure for sectioning frozen brain tissue and collecting the slices. Serial coronal brain sections are collected using a cryostat, progressing rostrally to caudally, at a thickness of 50 μm. Sections are free-floating in a solution of PBS containing 0.01% sodium azide (NaN_3) to prevent microbial growth.

Note: To accurately target the LC, tissue orientation is guided morphologically using the Paxinos Brain Atlas,[2] focusing on the region between the hereby reported coordinates (Interaural −1.54 mm, Bregma −5.34 mm and Interaural: −2.00 mm, Bregma: −5.80).

⚠ CRITICAL: To identify the structure and ensure the area is not missed, it is recommended to begin sectioning slightly before the indicated coordinates.

14. Sectioning the Locus Coeruleus (LC).
    a. Position the brain on the metallic holder with the cerebellum facing down and the olfactory bulbs facing up.
        i. Secure it firmly and embed it in OCT compound, applying it layer by layer.
        ii. Ensure that each layer is fully frozen before adding the next to maintain structural integrity.

⚠ CRITICAL: The brain must be positioned perfectly perpendicular to the metallic holder before fully embedding.

        iii. Once fully covered, place the metallic support in the cryostat and begin sectioning rostrally to caudally.
    b. Trim the brain to quickly remove excess tissue from regions anterior to the area of interest.
        i. Continue until you are just before the LC.
        ii. Continue trimming until just ***before*** reaching the LC; this area is ideally identifiable by the characteristic morphology of the brain (Interaural −1.22, Bregma −5.02) as illustrated in Figure 1.

Note: A key anatomical marker for orientation is the fourth ventricle, located just medial to the LC. Because of their close proximity, the width and shape of the fourth ventricle serve as reliable morphological cues for accurately locating the LC during sectioning (**black arrow** in Figure 1).

Note: Another useful reference point is the inferior colliculi, which appear as two rounded structures on the dorsal surface of the midbrain (sort of resembling a pair of eyeglass lenses placed side by side, red arrow in Figure 1).

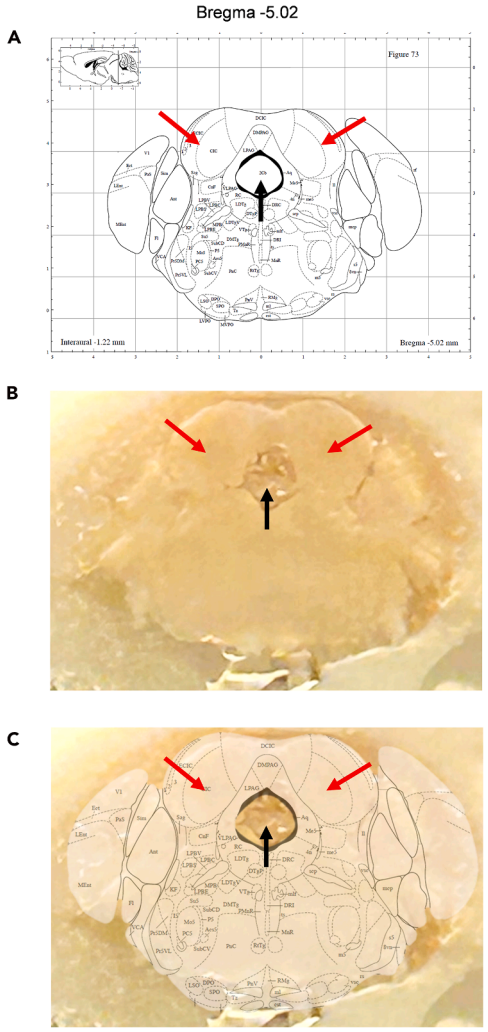

Figure 1. Representative image of the brain in a region slightly anterior to the locus coeruleus (LC), where sectioning is ideally initiated

(A) shows the reference Paxinos Brain Atlas image for this region, corresponding to bregma −5.02.

(B) highlights the characteristic brain morphology that aids in its identification.

(C) presents an overlay of Panels A and B to facilitate the recognition of anatomical landmarks. In all three panels, the black arrow points to the fourth ventricle, while the red arrows indicate the inferior colliculi in the right and left hemispheres. These structures can be used as reliable reference points for anatomical orientation of the LC area.

c. Set the cryostat to a section thickness of 50 μm and begin collecting serial coronal brain sections. Under these settings, the LC typically spans a minimum of 5 to a maximum of 7 consecutive sections (250–350 μm).
d. As each slice is collected, transfer it immediately into a 12-well plate pre-filled with PBS containing 0.01% sodium azide (NaN_3) to prevent microbial growth.
e. Continue sectioning slightly beyond the LC (~Interaural −2.08 mm, Bregma −5.88 mm) to ensure the entire region has been collected.

Immunohistochemistry (major step 1)

Timing: 3 days

The section below details the staining protocol of brain sections for quantifying tyrosine hydroxylase positive (TH+) neurons (phenotypic marker) and cresyl violet stained cells (Nissl staining, structural marker) in LC.

Sections are free-floating; perform all steps in a 12-multiwell plate.

Day 1

15. Transfer all the slices obtained by cryostat cutting in the first well of a 12-multiwell plate pre-filled with PBS.
16. Wash sections in PBS 3 times for 3 min each. Transfer the sections in a new well each time; this step will be useful to dissolve the small layer of OCT surrounding each slice.
17. In a new well, incubate with 3% H_2O_2 in PBS for 10 min at 20°C–22°C to inhibit endogenous peroxidase activity.
18. Wash sections in PBS twice for 3 min each time.
19. Incubate sections with blocking solution (PBS containing 0.3% Triton X-100, PBS-T and 2% BSA) for 30 min at 20°C–22°C.
20. Incubate sections with anti-TH antibody (1:750; Millipore, mab318) in PBS-T added with 1% BSA for 16 h at 4°C [Troubleshooting n.1, Troubleshooting n.2].

Day 2

21. Wash sections in PBS 4 times for 3 min each. Incubate sections with HRP-conjugated secondary antibody (GE Healthcare, 1:500 in PBS-T + 1% BSA) for 1 h at 20°C–22°C.
22. Wash sections in PBS 4 times for 3 min each.
23. Incubate each section with 3,3-Diaminobenzidine (DAB) tetrahydrochloride substrate kit (Abcam, ab64238).
    a. Carefully observe colour development and stop the reaction when the desired colour is obtained.
    b. Prepare DAB according to the manufacturer's directions: one drop (~30 µL) DAB Chromogen in 1.5 mL DAB substrate.
24. Wash sections in PBS 4 times for 3 min each.
25. Mount sections exhibiting the characteristic DAB signal onto gelatin-coated slides.

Note: Not all sections will display staining, as tissue was on purpose sectioned starting slightly before and ending after the area of interest.

26. Dry sections on a plate heated to 50°C for 1 h.
27. Rinse the slides in H_2O to remove PBS salts that may affect the visualization of the slices under the microscope.
28. Dry sections on a plate heated to 50°C for 30 min.
29. Dehydrate sections by incubating for 16 h in a chloroform-alcohol mixture (1:1). This results in the reduction of sections thickness from 50 µm to approximately 10 µm.

Day 3: Nissl counterstaining

Note: Nissl staining is a procedure that uses cresyl violet to stain Nissl bodies in neurons.

30. Rehydrate slides.
31. Incubate with 0.1% Cresyl Violet for 2 min.

⚠ CRITICAL: Warm the cresyl violet solution to 37°C to improve staining.

(1) Define settings and trace region of interest.

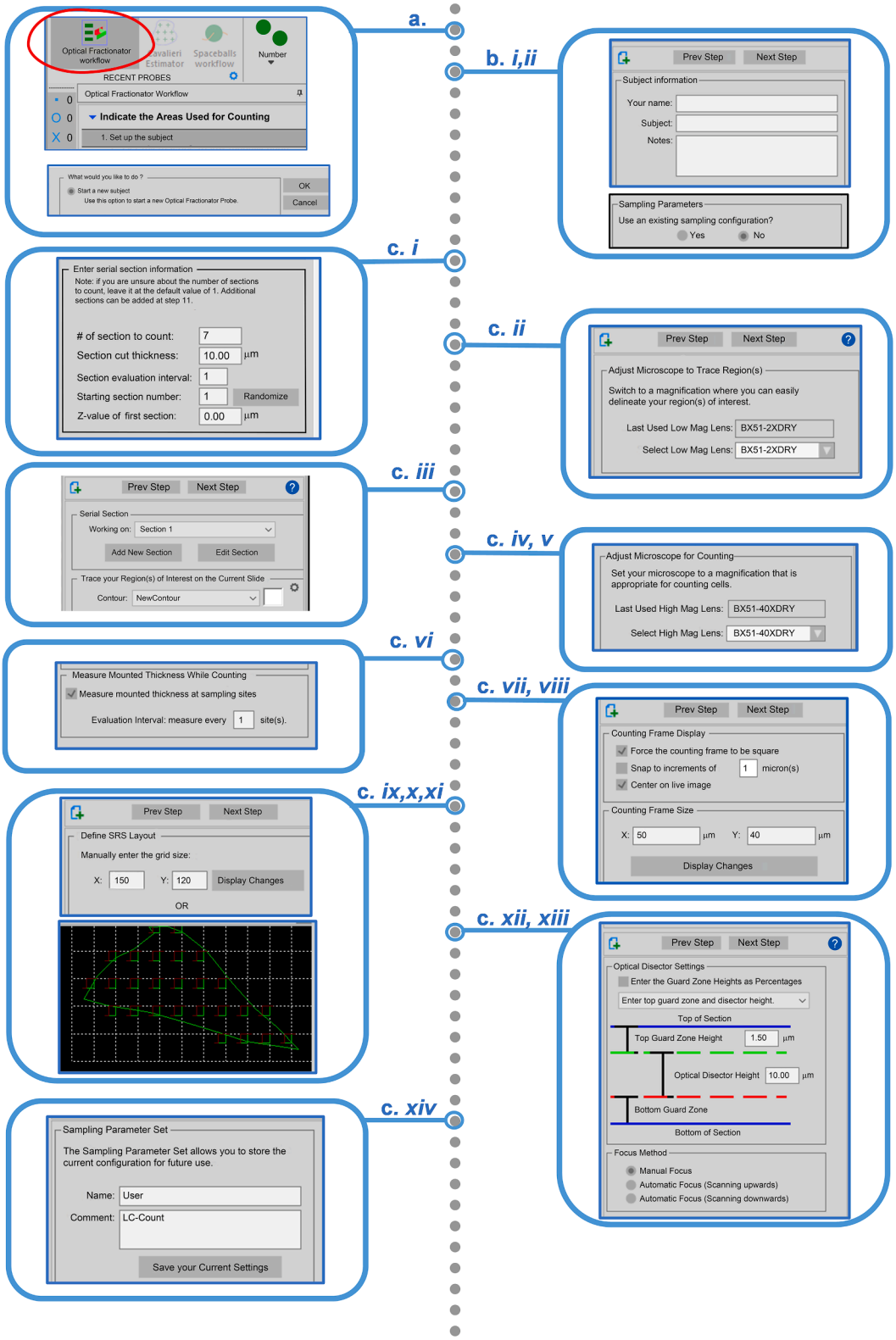

Figure 2. Workflow of stereological analysis using the Stereo Investigator software, including the major steps described in the protocol regarding the definition of settings and the outlining of the region of interest

Upon launching the software, the *Optical Fractionator workflow* is selected (a, red circle). In the "*Areas Used for Counting*" section, the user begins by clicking on *Start a new subject* (a) and enters the quantification name and staining type (b. *i, ii*). Under *Probe Configuration*, the number of sections to analyze is specified, followed by setting section thickness to 10 μm (c. *i*). The 2.5x objective is used to identify the region of interest, selecting the appropriate section and contour type (left and right), ensuring accurate delineation of bilateral structures (c. *ii,iii*). Next, the 40x objective is selected for high-resolution counting (c. *iv,v*). Users must enable *Measure mounted thickness at sampling sites*, setting the evaluation interval to 1 to account for tissue shrinkage (c. *vi*). The *Counting Frame Size* is set to 50 × 40 μm (c. *vii,viii*), ensuring ~1–3 cells per frame, while the grid spacing is manually defined (150 μm × 120 μm) to limit the number of frames to fewer than 50 per region structures; an example of final grid is reported (c. *ix,x,xi*). The *Dissector Thickness* is defined with a 1.5 μm top guard zone and a 20 μm dissector height (c. *xii,xiii*). Finally, the sampling parameter set (e.g., "LC_Count") is named and saved to complete the setup (c. *xiv*).

32. Rinse slides in distilled water.
33. Dehydrate slides through sequential incubations for few seconds in EtOH 95% and EtOH 100%.
34. Clear in xylene.
35. Mount slides using a xylene based mounting media (Eukitt mounting medium, Bio-Optica) and seal with coverslips.

⚠ CRITICAL: Avoid air bubble formation when placing the coverslip.

Image acquisition and cell count (major step 2)

Timing: 1 h

This section outlines the procedure for tracing the regions where the counts will be performed (1), and for performing cell counts as part of the estimation of cell number with a commercially available stereology system (2). To do this, the Optical Fractionator probe within the Stereo Investigator software will be used. The key steps are detailed in the numbered list below and shown in Figures 2 and 3.

36. Define settings and trace region of interest (Figure 2).
    a. Upon opening of the Stereo Investigator software, set the Optical Fractionator workflow.
    b. Indicate the Areas Used for Counting section. First, click on Start a new subject.
        i. Insert the name of the type of quantification (user's name).
        ii. Insert the subject (type of staining: e.g., TH, Nissl).
    c. Define Probe Configuration Section. Scroll down the window until #sections to count and type the number of slices per animal to be taken in consideration.
        i. Type the thickness (10 μm), then click on Next Step.
        ii. Choose the 2.5x magnification lens to draw the region of interest, then click on Next Step.
        iii. Draw the region of interest. Choose the section (Working on) and the type of contour needed (Select Contour, left and right contour,) and trace the edges of your structures. Right click and Close contour to close the region; click on Next Step. Ensuring bilateral representation: it is crucial that each section includes both the left and right hemispheres.

⚠ CRITICAL: *The traced region must be as precise as possible to avoid including any surface area outside the intended region of interest.* [Troubleshooting n.3]

        iv. Choose the high magnification lens that will be needed for counting cells (40x). Then, change the selected objected on the microscope manually; click on Next Step.
        v. Scroll down the window and select the case Measure mounted thickness at sampling sites.
        vi. Choose the evaluation interval, which represents how often we have to measure the thickness of our z. The best and more accurate choice is #1. This is mandatory because of the shrinkage due to dehydration; click on Next Step.

(2) Perform Counting.

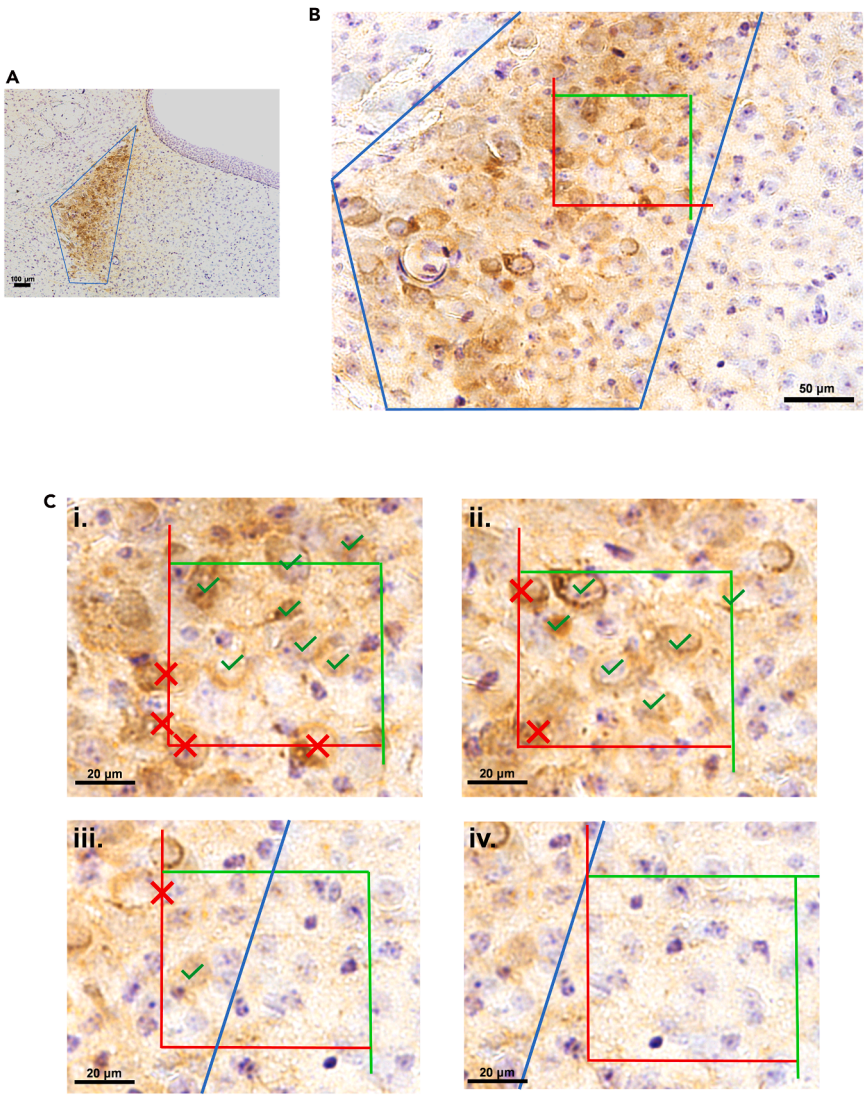

Figure 3. Representative figure illustrating key aspects of performing cell counts

(A) Shows an example of a traced region selected for analysis. Scale bar is 100 µm.

(B) Shows a high-magnification view of the traced area, highlighting the counting frame (previously defined in Figure 2C,vii,viii,xi). The frame includes green inclusion lines and red exclusion lines. Cells should be counted based on the following general rules: count all cells that are fully within the counting frame or touching the green inclusion lines. Axonal and dendritic processes should not touch or cross the red exclusion lines. Scale bar is 50 µm.

(C) Shows examples of different scenarios encountered during counting. Panels (i) and (ii) show proper alignment of the counting frame within the traced area; neurons with a green check should be counted while neurons with a red cross should not; panels (iii) and (iv) depict misaligned placements. Scale bar is 20 µm.

vii. In the Counting Frame Size field, enter the dimensions of the square used for object counting which will be 50 × 40 µm.

viii. By clicking on Display Changes, it will be possible to visualize the actual size of the counting frame.

ix. When determining the appropriate frame size, ensure that each frame contains approximately 1 to 3 cells. Once set, click on Next Step to proceed.

x. Click on Manually Enter Grid Size to define the distance between counting frames.

xi. Set the grid spacing to 150 μm (X-axis) and 120 μm (Y-axis). Click on Display Changes to visualize the grid layout and confirm the spacing.

Note: It is important to adjust the grid size so that fewer than 50 counting frames are generated per region—this ensures efficient and accurate data collection. Once set, click on Next Step to proceed.

xii. Next, set the Dissector Thickness parameters. For sections mounted in xylene-based medium, use the following standard settings: top Section/Top Guard Zone must be 1.50 μm, and Optical Dissector Height 10 μm.
xiii. Once set, click on Next Step to proceed.

⚠ CRITICAL: These Dissector Thickness parameters ensure a working range that is slightly greater than the actual slice thickness after dehydration.

xiv. Enter the name of the sampling parameter set (e.g., LC_Count); scroll down and click on Save your current settings.

37. Perform counting (Figure 3).
    a. Click on the name of the region that you wish to analyze (an example of traced area is shown in Figure 3A). Select your preferred symbol for marking counted neurons from the toolbar on the left. Then, scroll down and click the green arrow to begin counting.
    b. When the prompt Focus top of section appears, adjust the microscope focus until the surface of your tissue slice just begins to blur—this indicates the top of the section. Once focused, click *OK*.
    c. Repeat the process when prompted to Focus bottom of section. Once the focal thickness is set, a green scale will appear on the right side of the screen, indicating the valid counting (working) interval.
    d. You are now ready to begin counting. The screen will appear as shown in Figure 3B. Count all cells that are: fully within the square or touching the green inclusion lines. Adhere to the following neuron selection criteria:
        i. Appearance: Neurons should appear full and dark.

⚠ CRITICAL: Neurons should not appear in sharp focus immediately; if this happens, it may indicate the presence of staining artifacts.

        ii. Size consistency: selected neurons should exhibit a relatively uniform soma size across all tissue sections, soma dimension is between 20 and 40 μm.[3,4]
        iii. Morphology: The soma (cell body) must be included within the green square for at least two-thirds of its area. Axonal and dendritic processes should not touch or cross the red border of the square.
        Do not count cells that touch the red exclusion lines and/or fall outside the counting frame.
        An example of different scenarios where the neuron selection criteria apply is shown in Figure 3C.
    e. The next drawn section should be proceeded with by clicking on Next Step. This step must be repeated for each drawn section [Troubleshooting n.4]. A minimum of 6 and a maximum of 7 slides should be counted. [Troubleshooting n.5].
    f. Once the counting is finished, click on the Counting Frame icon to review the counts for each slide.
        i. Select I have finished counting.
        ii. To proceed with stereological quantification, select all entries and click on View Results.

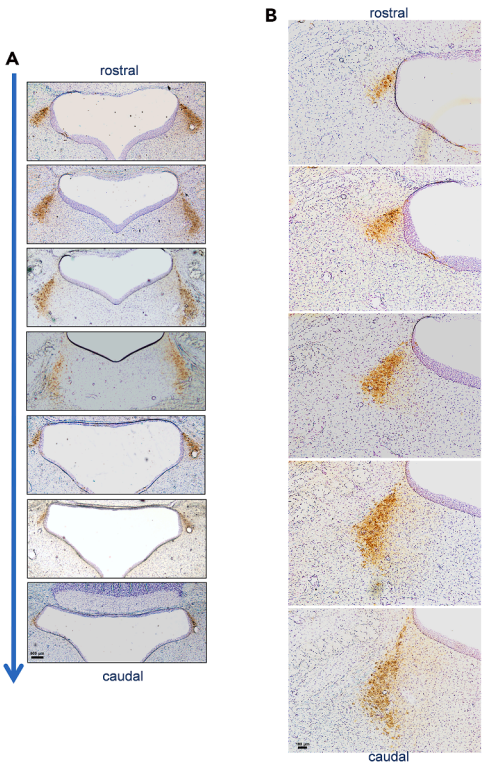

Figure 4. Representative images of mouse LC sections

LC sections were stained as explained in the Immunochemistry Sections (Major Step 1) for tyrosine hydroxylase (TH), and are stacked coronally, from rostral to caudal level in both low-magnification (A) and corresponding high-magnification (B). Note how the width of the fourth ventricle can serve as a morphological landmark for orientation during and after brain sectioning. These images can be used as a guide to help the user in orientating during staining, area tracing and counting process. Scale bar in A is 500 μm, scale bar in B is 100 μm.

iii. Define the section interval to correspond with the sampling rate. In this case, the original section thickness was 50 μm; however, since the dehydration step reduces the brain slice thickness by a factor of five, the interval should be set to 10 μm.

⚠ CRITICAL: Avoid relying on the automatic thickness measurement proposed by the software: even subtle variations in thickness (e.g., caused by factors such as mispositioned coverslips, slight excess of xylene, or variations in slice thickness) can lead to inaccurate thickness measurements and ultimately result in unreliable neuron count estimates.

g. When the metadata summary window appears, click Export to Excel to generate and view your final results.

EXPECTED OUTCOMES

Stereological counting is a gold standard for quantifying neuronal populations in brain areas.[5] To our knowledge, our protocol is the first to provide a method for quantifying neuronal populations in the small but critical area of the LC. A key step of the protocol, achieved via brain sectioning and immunohistochemistry, is the generation of high-quality images. Expected outcome includes images as displayed in Figure 4: these images exemplify optimal conditions for the purpose of quantification. Suboptimal images can stem from several factors, such as asymmetry (this happens when the sections lack symmetry between the two hemispheres, which is crucial for accurately counting neurons in both), Presence of Folds which can obscure individual nuclei, making them indistinguishable, and Faint Staining which can prevent clear differentiation between neurons and the surrounding parenchyma.

All these issues can significantly influence neuron counts. Therefore, if any of these situations occur, the procedure should be repeated.

Using the presented method, the expected number of TH+ NE neurons in the LC of adult mouse is 1377 ± 99 (mean ± SEM). Our associated primary research manuscript in WT mice (C57BL6/N strain) found 1390 ± 100 neurons at 1 month of age, 1377 ± 99 neurons at 6 months of age and 1353 ± 135 neurons at 12 months of age. Significant differences can be found in pathological conditions that affect this brain area, such as PD.[1] In the *Prkn*R275W mouse model of PD, we found a significant reduction in TH+ neurons of LC at all ages tested: 1040 ± 55 at 1 month, 1048 ± 99 at 6 months, 912 ± 71 at 12 months. We expect a degeneration of LC neurons in other mouse models of PD and in murine models of Lewy Body Dementias,[6] AD[7] and other tauopathies.[8] Another consideration concerns potential differences in the number of neurons in the LC across different mouse strains. Different mouse strains (e.g., C57BL/6J, BALB/c, DBA/2J, 129S strains) have distinct genetic compositions. Genes involved in neurogenesis, neuronal migration, differentiation, and apoptosis can vary, leading to changes in neuron counts. Some strains might have proportionally larger or smaller specific brain regions, which could correlate with different numbers of neurons in those areas.[9,10] Moreover, certain strains might be more prone to age-related neuron loss, influencing neuron counts in older animals.[11,12] Thus, mouse strain matters for neuron number, and this factor should also be considered for LC neurons. Nissl staining can be used as a counterstain to provide anatomical context and help localize stained cells.

In conclusion, our protocol offers a method for quantifying TH+ neuronal populations in the LC. The protocol is proposed as a valuable tool for studies aimed at investigating LC degeneration in aging or pathological conditions, as well as for evaluating the effects of neuroprotective therapies.

QUANTIFICATION AND STATISTICAL ANALYSIS

Considering the potential biological variability, we suggest LC counting be performed on mouse cohorts including a minimum of n=10 animals for each genotype/age/treatment. Data from stereological counts are normally distributed, thus they can be analyzed with parametric statistical tests. Statistical analysis of data can be performed using Student's *t* test or One-Way ANOVA, followed by appropriate post-hoc comparisons. Two-Way ANOVA can be used to examine the interaction of two distinct independent variables (for example, age and genotype, age and treatment) on LC neuron numbers.

LIMITATIONS

This stereology protocol provides unbiased estimates relying on shape and distribution of the cells, rather than exact counts. Sampling errors may arise if the tissue is not adequately or evenly sampled. Successful implementation requires adequate training of the operator, as poor technique or lack of experience can compromise data accuracy. These factors must be carefully considered when interpreting stereological data.

TROUBLESHOOTING

Problem 1

During immunostaining, brain slices collected from the well after incubating for 16 h with the primary antibody may adhere to each other, rendering them difficult to separate and unsuitable for further processing.

Potential solution

- Antibody solution volume: the likelihood of the slices sticking together increases as the incubation solution volume decreases. If this happens, consider increasing the volume of the incubation solution.
- Gentle swirling: try gently swirling the well to see if the movement helps them detach.

- Fine paintbrush or wide-bore pipette tip: carefully use a fine, soft paintbrush or a pipette tip with a wide opening to *gently* nudge them apart.

Problem 2

Bacterial and Mold Growth in Tissue Slices.

Potential solution

Contaminated slices cannot be used anymore. For long-term storage, remember to treat slices with a cryopreserving solution immediately after cryostat sectioning. This crucial step helps minimize the risk of bacterial and mold contamination.

Problem 3

When tracing the section contours, discrepancies in shape between the left and right sides are observed.

Potential solution

- Uneven Sections: At least 6 valid sections containing LC TH+ neurons are required for reliable quantification.
- Asymmetrical Slices: Due to cutting variability, slices may not be perfectly symmetrical. When tracing the LC, select the most appropriate matching section from the opposite hemisphere, even if it is from a different slice. This approach will allow pairing LC areas based on anatomical similarity rather than strict slide order. Keep in mind that counts will be averaged across all sections.

Problem 4

When moving from one section to the other, the system crashes.

Potential solution

Joystick Movement Warning: Avoid rapid, simultaneous motion of the joystick in both the vertical (up/down) and horizontal (left/right) directions, as this may cause the software to crash. Move slowly and in one direction at a time to ensure stability.

Problem 5

There is considerable variability in the counts obtained, which cannot be attributed to the physiological variability of the mice. The numbers are either too high or too low compared to the standard.

Potential solutions

- Slices: re-evaluate your entire protocol to ensure that the slices have been adequately prepared. To fully capture the LC, we recommend beginning your sectioning slightly before the LC anterior-most boundary and continuing until slightly past its posterior-most boundary. For precise stereological guidance, refer to an atlas for the specific range (e.g., Bregma: from −5.40 mm to −5.68 mm; lateral: ±1.0 mm from midline, dorsoventral: −3.0 mm to −3.8 mm from the dura.
- New model: If the mouse model that you are studying has not been analyzed from this perspective before, the number of LC neurons might differ from the expected values. In such cases, perform biological replicates to confirm whether the data are consistent across other mice of the same age, genotype, and treatment characteristics.
- Minimum Area Criteria: The LC tracing should encompass from 5 to 10 grid squares. If the area exceeds 14 squares, it is likely that the contour was inaccurately traced and overestimated: in this case it should be redrawn. If the area falls below 5 squares, the contour may have missed relevant portions of the structure and should be reviewed for completeness.
- Same-Day Counting: Perform as many counts as possible on the same day to reduce inter-day variability and potential bias.

- **Consistency Across Counters**: All counts should be performed by the same individual to minimize inter-operator variability and ensure consistency across samples.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jenny Sassone (sassone.jenny@hsr.it).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Letizia Zanetti (lz3041@cumc.columbia.edu).

Materials availability

The presented protocol can be executed with commercially available materials and does not require any original or customized materials.

Data and code availability

The protocol includes neither datasets nor computer codes.

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AUTHOR CONTRIBUTIONS

L.Z., M.R., and J.S. wrote the paper (review and editing and original draft). F.V. and M.M. were involved in the review and editing. E.M., C.M., and G.D.G. performed the experiments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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