Protocol

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Assessment of neurodegeneration and neuronal loss in aged 5XFAD mice

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SUMMARY
Neuronal loss resulting from progressive neurodegeneration is a major pathological feature of Alzheimer’s disease (AD). Here, we present a protocol to detect neurodegeneration, neuronal apoptosis, and neuronal loss in 5XFAD mouse strain, which is a well-established model for interrogating the molecular mechanism of neuronal death in AD. This protocol describes the use of the neurodegenerative marker Fluro-Jade C, cleaved caspase-3 immunofluorescent staining and Nissl staining for the analysis of neurodegeneration and neuronal loss in 5XFAD mice. For complete details on the use and execution of this protocol, please refer to Zhang et al. (2021).

BEFORE YOU BEGIN
This protocol describes the specific steps to analysis (1) neurodegeneration with the method of Fluro-Jade C (FJC) staining, (2) neuronal apoptosis using immunofluorescent staining with anti-cleaved caspase-3 antibody and (3) neuronal loss using Nissl staining combined with immunofluorescent staining with anti-NeuN antibody, in the brain slices of 9-month-old 5XFAD mice and age-matched wide-type (WT) control mice. In our experience, neurodegeneration can be analyzed using 6–9 months old 5XFAD mice. However, using 9–12 months old 5XFAD for neuronal apoptosis and neuronal loss analysis would be better, because the signal of cleaved caspase-3 is hard to be detected in the brain of younger 5XFAD mice.

Before the staining, you need to prepare the following animals and solutions (refer to “Materials and equipment” for detailed recipe.):

9 months old 5XFAD male mice and WT control male mice.

Pre-chilled 1× PBS for the perfusion of mouse brains.

4% paraformaldehyde (PFA) fixing solution.

Anti-freezing solution for the storage of mouse brain sections.

0.01% Fluro-Jade C stock solution for FJC staining.

0.06% potassium permanganate solution for FJC staining.
80% ethanol containing 1% NaOH for FJC staining.

Blocking buffer for immunofluorescent staining.

PBST washing buffer for immunofluorescent staining.

0.5% Cresyl violet solution for Nissl staining.

95% ethanol containing 0.7% glacial acetic acid de-staining solution for Nissl staining.

Prepare coronal sections

© Timing: 4–5 days

1. Brain samples preparation
   a. Anesthetize mice with Ketamine/Xylazine (Ketamine: 50–80 mg/kg; Xylazine: 5–10 mg/kg) via intraperitoneal injection. Toe pinch reflection is recommended to monitor the depth of anesthesia. It usually takes 5–10 min to complete anesthesia.
   b. Perfuse the mice with ice-cold 1× PBS via left ventricle.
   c. Dissect out the whole brain carefully, and then put the brain into a 15 mL centrifuge tube containing 4% PFA over night with gentle shaking on an orbital shaker at a speed of 50 rpm in cold room (4°C–8°C).

2. Dehydrate the brain samples
   a. Next day, prepare 15% sucrose solution with 1× PBS freshly, then transfer the fixed brain to a 15 mL centrifuge tube containing 15% sucrose solution, keep the tube standing in cold room until the brain falls to the bottom of the tube, it usually takes 1 day.
   b. After the brain has fallen, typically on the third day since beginning the protocol, transfer the brain to another 15 mL centrifuge tube containing freshly prepared 30% sucrose dissolved in 1× PBS, still keep the tube standing in cold room until the brain falls to the bottom of the tube, this step will take 1–2 days.

   Note: Use filtered or autoclaved 1× PBS solution with 0.1% sodium azide to prepare sucrose solutions for long-term storage of the brain.

   c. Store dehydrated brain at −80°C until ready to prepare frozen sections.

   Note: The dehydrated brain can be stored at −80°C for 1 year.

3. Make brain coronal sections
   a. Section dehydrated brains for serial 30–50 μm coronal sections using a Thermo NX50 freezing microtome.
   b. Store coronal section in anti-freezing solution at −20°C before staining.

   Note: Both mounted and free-floating sections are suitable for the following staining procedures. Free-floating sections are used in the following protocols.

△ CRITICAL: The usage of Ketamine/Xylazine anesthetic and the performance on mice should conform to the relevant regulatory standards of Institutional Animal Care and Use Committee (IACUC).
### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Rabbit anti-Cleaved caspase-3 (1:100) | Millipore | Cat# AB3623; RRID:AB_91556 |
| Guinea pig anti-NeuN (1:500) | Millipore | Cat# ABN90P; RRID:AB_2341095 |
| Donkey anti-rabbit IgG (H+L) Alexa Fluor® 594 (1:1000) | Jackson ImmunoResearch | Cat# 711-585-152; RRID:AB_2340621 |
| Donkey anti-guinea pig IgG (H+L) Alexa Fluor® 488 (1:1000) | Jackson ImmunoResearch | Cat# 706-545-148; RRID:AB_2340472 |
| Chemicals, peptides, and recombinant proteins |        |            |
| Fluoro-Jade C | Millipore | Cat# AG325 |
| Cresyl Violet | Sigma-Aldrich | Cat# C5042 |
| DAPI | Sigma-Aldrich | Cat# D9542 |
| Sucrose | Sigma-Aldrich | Cat# V900116 |
| PVP-40 | Sigma-Aldrich | Cat# PVP40 |
| Ethylene Glycol | Sigma-Aldrich | Cat# 324558 |
| Glacial acetic acid | Sigma-Aldrich | Cat# A6283 |
| Potassium permanganate | Sigma-Aldrich | Cat# 31404 |
| NaOH | Sigma-Aldrich | Cat# 221465 |
| PBS | Sigma-Aldrich | Cat# P3813 |
| Tween 20 | Sigma-Aldrich | Cat# P1379 |
| Tissue-Tek O.C.T. Compound | Sakura | Cat# 4583 |
| Normal Donkey Serum | Jackson ImmunoResearch | Cat# 071-000-121 |
| Neutral balsam | Solarbio | Cat# G8590 |
| 16% Formaldehyde | Cell Signaling Technology | Cat# 12606 |
| Ketamine | R&D | Cat# 3131/50 |
| Xylazine | Selleck | Cat# S2516 |
| Experimental models: Organisms/strains |        |            |
| Mouse: B6.Cg-Tg(APP569SwFlLox,PSEN1* M146L*L286V/6795Vas/Mni/jax (5XFAD) | The Jackson Laboratory | Cat# 34848-JAX; RRID:MMRRC_034848-JAX |
| Mouse: C57BL/6J | The Jackson Laboratory | Cat# JAX:000664; RRID:IMSR_JAX:000664 |
| Software and algorithms |        |            |
| Fiji-ImageJ | National Institute of Health | https://imagej.net/Fiji; RRID:SCR_003070 |
| GraphPad Prism 6 | GraphPad | https://www.graphpad.com/scientific-software/prism/; RRID:SCR_002798 |
| Other |        |            |
| Olympus BX51 microscope | Olympus | BX51 |
| Leica TCS SP8 confocal microscope | Leica | SP8 |
| Thermo FX500 freezing microtome | Thermo Fisher Scientific | NX50 |
| Gelatin-coated microscope slides | FD Neurotechnologies | Cat# PO101 |
| Fluorescence Mounting Medium | Dako | Cat# S3023 |

## MATERIALS AND EQUIPMENT

- 4% PFA fixing solution:

| Reagent       | Final concentration | Amount or volume |
|---------------|---------------------|------------------|
| 16% Formaldehyde | 4%                  | 10 mL            |
| 1x PBS        | n/a                 | 30 mL            |
| Total         | n/a                 | 40 mL            |
Note: 4% PFA solution need to be prepared freshly.

- **Anti-freezing solution:**

| Reagent               | Final concentration | Amount or volume |
|-----------------------|---------------------|------------------|
| NaH2PO4·2H2O         | 0.02 M              | 1.560 g          |
| Na2HPO4·12H2O        | 0.08 M              | 3.581 g          |
| PVP-40                | 1%                  | 5 g              |
| Sucrose               | 30%                 | 150 g            |
| Ethylene glycol       | 30%                 | 150 mL           |
| ddH₂O                 | n/a                 | To 500 mL        |
| Total                 | n/a                 | 500 mL           |

Note: Anti-freezing solution can be stored at room temperature (20°C–25°C) up to 2 weeks.

- **0.06% potassium permanganate:**

| Reagent                  | Final concentration | Amount or volume |
|--------------------------|---------------------|------------------|
| Potassium permanganate   | 0.06%               | 0.015 g          |
| ddH₂O                    | n/a                 | To 25 mL         |
| Total                    | n/a                 | 25 mL            |

Note: 0.06% potassium permanganate solution need to be prepared freshly.

- **Blocking buffer:**

| Reagent                        | Final concentration | Amount or volume |
|--------------------------------|---------------------|------------------|
| Normal donkey serum            | 5%                  | 0.5 mL           |
| BSA                            | 1%                  | 0.1 g            |
| 1× PBS                         | n/a                 | To 10 mL         |
| Total                          | n/a                 | 10 mL            |

Note: Blocking buffer can be stored at 4°C up to 2 days.

- **PBST washing buffer:**

| Reagent   | Final concentration | Amount or volume |
|-----------|---------------------|------------------|
| Tween 20  | 0.2%                | 0.1 mL           |
| 1× PBS    | n/a                 | To 50 mL         |
| Total     | n/a                 | 50 mL            |

Note: Washing buffer can be stored at room temperature up to 1 week.

- **De-staining solution:**

| Reagent         | Final concentration | Amount or volume |
|-----------------|---------------------|------------------|
| Glacial acetic acid | 0.7%              | 1.4 mL           |
| 100% ethanol    | 95%                 | 190 mL           |
| ddH₂O           | n/a                 | 8.6 mL           |
| Total           | n/a                 | 200 mL           |

Note: De-staining solution can be stored at room temperature for 1 month.
- **80% ethanol containing 1% NaOH:**
  - Dissolve 0.25 g NaOH with 5 mL distilled water.
  - Mix above NaOH solution with 20 mL 100% ethanol.
  - Store this solution at room temperature for up to 1 day.

- **Fluro-Jade C staining solution:**
  - Dissolve 10 mg Fluro-Jade C in 100 mL distilled water to make 0.01% stock solution.
  - Dilute 0.1 mL glacial acetic acid with 100 mL distilled water to make 0.1% acetic acid solution.
  - Just before use, dilute 0.01% FJC stock solution in 0.1% acetic acid solution to make 0.0001% working solution with a ratio of 1:100.
  - Store 0.01% FJC stock solution and 0.1% acetic acid solution at 2°C–8°C for up to 3 months.

- **Cresyl violet solution:**
  - Dissolve 1 g Cresyl violet acetate in 200 mL distilled water.
  - Add 500 μL glacial acetic acid and filter the solution with filter paper.
  - Store 0.5% Cresyl violet solution at room temperature for up to 6 months.

⚠ **CRITICAL:** Glacial acetic acid and NaOH are corrosive, and PFA is toxic in contact with skin and if inhaled. Always wear gloves, mask and lab coat to protect yourself, and handle glacial acetic acid and PFA under a chemical hood.

**Alternatives:** In principle, the reagents and resources listed in the key resources table can be replaced with equivalent items from other suppliers; however, the impact of alternative reagents on protocol performance has not been tested.

**Alternatives:** This protocol uses Olympus BX-51 microscope and Leica TCS SP8 confocal microscope for imaging. Alternative microscopes can be used as well.

### STEP-BY-STEP METHOD DETAILS

**Fluro-Jade C staining of degenerated neurons**

© **Timing:** 2–3 h

**Rationale:** Fluro-Jade C staining is able to stain all degenerating neurons, regardless of specific insult or mechanism of cell death. It is ideal for localizing not only degenerating nerve cell bodies, but also distal dendrites, axons and terminals, due to greatest signal to background ratio, as well as the highest resolution. Sections labeled with FJC can also be immuno-stained to achieve multiplexed labeling.

1. **Pre-treating free-floating brain sections**
   a. Wash free-floating brain sections with 1× PBS 5 times, 5 min for each time, to remove anti-freezing solution completely.
   b. Pre-treat sections as indicated below:

| Step | Solution                              | Time of immersion |
|------|---------------------------------------|-------------------|
| 1    | 80% ethanol containing 1% NaOH        | 5 min             |
| 2    | 70% ethanol                           | 2 min             |
| 3    | 50% ethanol                           | 2 min             |
| 4    | 30% ethanol                           | 2 min             |
| 5    | 10% ethanol                           | 2 min             |
| 6    | ddH2O                                 | 2 min, twice      |
| 7    | 0.06% potassium permanganate          | 10 min            |
| 8    | ddH2O                                 | 2 min, twice      |
Note: Pre-treating should be performed in the wells of a 24- or 12-well cell culture plate for free-floating brain sections. Brain sections will shrink in 0.06% potassium permanganate solution and expand in ddH₂O, but will recover to normal shape in PBS. Keep staining regardless of shape change of the brain sections.

2. Performing FJC staining
a. Dilute DAPI stock solution (0.1 mg/mL) in 0.0001% FJC working solution at a ratio of 1:1000.
b. Stain brain sections with 0.0001% FJC working solution containing DAPI dye for 20 min.
c. Wash brain sections with ddH₂O for 3 times to remove extra FJC solution.
d. Recover brain sections in 1/3 PBS.

Pause point: Sections can be stored in 1/3 PBS for 0.5–1 h before mounting.

e. Mount brain sections on slides with Fluorescence Mounting Medium.
f. Put cover glass on the slides and push off bubbles and remove extra mounting medium.
g. Dry the mounted slides in the dark overnight (12–14 h) at room temperature.

Note: This protocol refers to a wet mount media, and nail polish should be used to seal coverslips. The mounted slides can be stored at 4°C for 1 month before imaging.

3. Quantification of degenerated neurons
a. Take pictures of degenerated neurons in cortex and hippocampus using an Olympus BX51 microscope with 20X objective lens (Figure 1). Use filter cube WU-#1 (dichroic mirror DM400, excitation filter BP330-385, barrier filter BA420) to visualize DAPI, and use filter cube WB-#2 (dichroic mirror DM500, excitation filter BP450-480, barrier filter BA515) to visualize FJC. For each mouse brain sample, three to five serial sections should be stained, and entire brain region should be pictured for quantification.
b. Open pictures with Fiji-ImageJ software, and use modules of “Measure” and “Analyze Particles” to calculate the density of degenerated neurons by measuring the brain area and counting the number of degenerated neurons respectively.
c. Statistically analyze the density (the number of FJC-positive neurons in given brain area (mm²)) of degenerated neurons among several groups using GraphPad Prism 6 software.

Immunofluorescent staining of apoptotic neurons

Timer: 2 days

Rationale: Apoptotic cells have the feature of enhanced activation of caspase-3, which is a specific executioner caspase in apoptosis. Anti-NeuN and anti-cleaved caspase-3 double staining is able to detect apoptosis specifically in neurons.

4. Wash brain sections with 1 x PBS for 3 times, 10 min for each time, to thoroughly remove anti-freezing solution.
5. Permeabilize sections in 0.3% Triton X-100 in 1 x PBS for 30 min.
6. Incubate sections in blocking buffer (5% normal donkey serum and 1% BSA in 1 x PBS) for 1 h at room temperature.
7. Prepare primary antibody dilutions of anti-cleaved caspase-3 (1:100) and anti-NeuN (1:500) with blocking buffer.
8. Incubate sections in primary antibodies overnight (12–14 h) on an orbital shaker at a speed of 50 rpm at 4°C.
9. Next day, wash sections with PBST washing buffer for 3 times, 10 min for each time.
10. Prepare secondary antibody solutions of donkey anti-rabbit IgG Alexa Fluor® 594 (1:1000) and donkey anti-guinea pig Alexa Fluor® 488 (1:1000), or fluorophores of your choice, with blocking buffer.

11. Incubate sections with secondary antibodies for 2 h at room temperature to visualize primary antibodies.

12. Wash sections with PBST for 3 times, 10 min for each time.

13. Incubate sections with 100 ng/mL DAPI solution to stain cell nuclei.

14. Mount brain sections on slides with Fluorescence Mounting Medium.

15. Put cover glass on the slides and push off bubbles and remove extra mounting medium.

16. Dry the mounted slides in the dark overnight (12–14 h) at room temperature.

**Note:** This protocol refers to a wet mount media, and nail polish should be used to seal coverslips. The mounted slides can be stored at 4°C for 1 month before imaging.

17. Image cleaved caspase-3 positive neurons using a Leica TCS SP8 confocal microscope (Figure 2). Typically, three to five serial sections per mice, and four to six fields per section need to be randomly pictured for quantification. 40X or 63X objective lens is recommended for imaging. Excitation and emission filters are used based on the fluorophores of your choice.

18. Statistically analyze the ratio of apoptotic neurons (number of apoptotic neurons/number of total neurons) among several groups using GraphPad Prism 6 software.
CRITICAL: The species of normal serum used for blocking buffer should be consistent with the species of your secondary antibodies. If you use goat anti-rabbit IgG and goat anti-guinea pig IgG secondary antibodies, you should use goat normal serum for blocking.

Nissl staining for neuronal density analysis

Timing: 4–5 h

Rationale: Cresyl violet acetate solution is able to stain Nissl substance in the cytoplasm of neurons. Nissl granules are purple, nuclei of glial cells and endothelial cells are slightly bluer than Nissl granules. Thus, the neuronal density in brain section can be measured by Nissl staining using Cresyl violet acetate.

19. Wash brain sections with 1/3 PBS for 3 times, 10 min for each time, to thoroughly remove anti-freezing solution.
20. Mount sections on gelatin-coated slides and allow them to dry at least for 1 h at room temperature.
21. Perform Nissl staining as shown below:

| Step | Solution                              | Time of immersion |
|------|---------------------------------------|-------------------|
| 1    | ddH₂O                                 | 10 s              |
| 2    | 0.5% Cresyl violet                     | 10 min            |
| 3    | ddH₂O                                 | 10 s              |
| 4    | 70% ethanol                           | 5 min             |
| 5    | 95% ethanol                           | 1 min             |
| 6    | 95% ethanol containing 0.7% glacial acetic acid | 1 min |
| 7    | 100% ethanol                          | 2 min, twice      |
| 8    | ethanol: xylene (1: 1)                | 5 min             |
| 9    | Xylene                                | 5 min             |

22. Mount brain sections on slides with neutral balsam.
23. Put cover glass on the slides and push off bubbles and remove extra neutral balsam.
24. Take pictures of brain sections using Olympus BX51 microscope under bright-field with 20X objective lens (Figure 3). For each mouse brain sample, three to five serial sections should be stained, and entire brain region should be pictured for quantification.
25. Open pictures with Fiji-ImageJ software, and use modules of “Measure” and “Analyze Particles” to calculate the neuronal density by measuring the brain area and counting the number of total neurons respectively.

26. Statistically analyze the neuronal density in given brain area (cells/mm²) among several groups using GraphPad Prism 6 software.

△ CRITICAL: Aged 5XFAD mice display significant neuronal loss in cortical layer 5 and subiculum of hippocampus according to literature (Oakley et al., 2006). However, significant neuronal loss can also be detected in the dentate gyrus of hippocampus in 9-month-old 5XFAD mice (Zhang et al., 2021). But the neuronal loss in dentate gyrus is hard to be analyzed via Nissl staining method due to compact localization of hippocampal neurons in dentate gyrus. Thus, we recommend to measure neuronal density in dentate gyrus using immunofluorescent staining with anti-NeuN antibody, combining with confocal imaging (Figure 4).

**EXPECTED OUTCOMES**

Neurodegeneration is a slowly progressive process during the development of Alzheimer’s disease in 5XFAD mice. Different experimenters will get different results on the numbers of degenerated neurons, apoptotic neurons or total neurons due to a lot of factors, such as the stage of the mice, the differences between staining reagents as well as the counting methods. However, it will not affect the conclusion on the difference of neuronal apoptosis or neuronal density between different groups, as long as you perform the staining and counting under the same condition and principle.

In our hands, the density of FJC-positive neurons is 30–40 cells/mm² in the cortex and hippocampus of 9-month-old 5XFAD mice. However, there is a few FJC-positive neurons, typically less than
1 cell/mm², in cortex and hippocampus of WT mice, which can serve as a negative control to confirm the reliability of your staining result (Figure 1).

In our experience, the ratio of cleaved caspase-3 positive neurons is 10%–15% in the cortex of 9-month-old 5XFAD mice, versus 2%–4% in 9-month-old WT mice. However, the percentage of cleaved caspase-3 positive neurons in dentate gyrus is relatively low, with a ratio of 1.5%–2% in 9-month-old 5XFAD mice and 0.2%–0.5% in age-matched WT mice.

9-month-old 5XFAD mice exhibit significant neuronal loss in cortical layer 5 and subiculum compared with WT control mice (Figure 3). The neuronal density in cortical layer 5 is about 1,500 cells/mm² in WT mice, but it drops to about 1,200 cell/mm² in 9-month-old 5XFAD mice. Similarly, the neuronal density in subiculum is about 2,500 cells/mm² in WT mice, but it significantly drops to about 1,000 cell/mm² in 9-month-old 5XFAD mice.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The experimenters should be blinded to genotype when taking pictures for microscopic analysis. For each mouse brain sample, three to five serial sections should be stained for each experiment, and entire brain region should be pictured for quantification. At least four to six fields per section need to be randomly pictured for quantification if the whole-brain imaging cannot be achieved due to the limitation of the microscopes. Comparison between two groups should be analyzed by unpaired two-tailed Student’s t-test and comparison among over three groups should be analyzed by one- or two-way ANOVA test depending on the treatment on mouse models.

**LIMITATIONS**

This protocol provides detailed methods to assess neurodegenerative phenotypes in aged 5XFAD mice from neurodegeneration, neuronal apoptosis to final neuronal loss. But this protocol does not evaluate glial markers of neurodegenerative processes (such as GFAP and Iba1 staining) and thus purely focuses on neuronal based analyses. Even though Fluro-Jade C is a specific neurodegenerative marker, the reason why Fluro-Jade C can specifically label degenerated neurons still unclear. In addition, using cleaved caspase-3 antibody to detect neuronal apoptosis remain controversial, as caspase-3 activation...
might be involved in synaptic function in the adult brain (Snigdha et al., 2012), thus methods that can detect neuronal apoptotic bodies and/or chromatin condensation, which are more proximal to death by apoptosis, need to be developed. Nissl staining is a widely used method to measure neuronal density. Nissl staining is very easy, cheap and timesaving, but the specificity of neuronal labeling is low as other cell types such as glial cells can also be stained by Cresyl violet, it takes some time to distinguish neurons from other cells when analyzing. By contrast, NeuN (or MAP2) staining is more specific than Nissl staining and contributes to more accurate results. All three staining methods are necessary when analyzing neuronal degeneration and death resulted from neuronal apoptosis, but only Fluro-Jade C and Nissl staining are required when you study other mechanisms of neuronal death.

TROUBLESHOOTING

Problem 1
The brain sections cannot recover to normal shape after FJC staining (step 2d).

Potential solution
Increase the recovering time in 1× PBS and change the 1× PBS for several times.

Problem 2
High green background of FJC staining when imaging using microscope (step 3a).

Potential solution
Wash brain sections with ddH2O for more times and extend the time for washing after FJC staining to remove extra FJC solution completely.

Problem 3
Weak or no signal of cleaved caspase-3 (step 17).

Potential solution
Increase the concentration of anti-cleaved caspase-3 antibody to 1:50.

Problem 4
Over- or under-staining of Nissl (step 21).

Potential solution
Always check the intensity of Nissl staining after submerging in 95% EtOH. If brain sections are over-stained, increase the time of immersion in differentiation solution (95% ethanol containing 0.7% glacial acetic acid) to 1.5–2 min. If brain sections are under-stained, decrease the time of immersion in differentiation solution to 10–30 s or skip differentiation.

Problem 5
There are many small holes in the brain slices when imaging (step 24).

Potential solution
Increase the time of dehydration of mouse brain in 30% sucrose solution. Insufficient dehydration usually causes the formation of ice crystal, which can destroy the normal structure of brain.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anning Lin (anninglin@nju.edu.cn).

Materials availability
This study did not generate new unique reagents.
Data and code availability
This study did not generate any unique data sets or code.

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AUTHOR CONTRIBUTIONS
L.Z. and J.L. optimized and conducted the experiments and wrote the manuscript; A.L. conceived the project and edited the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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