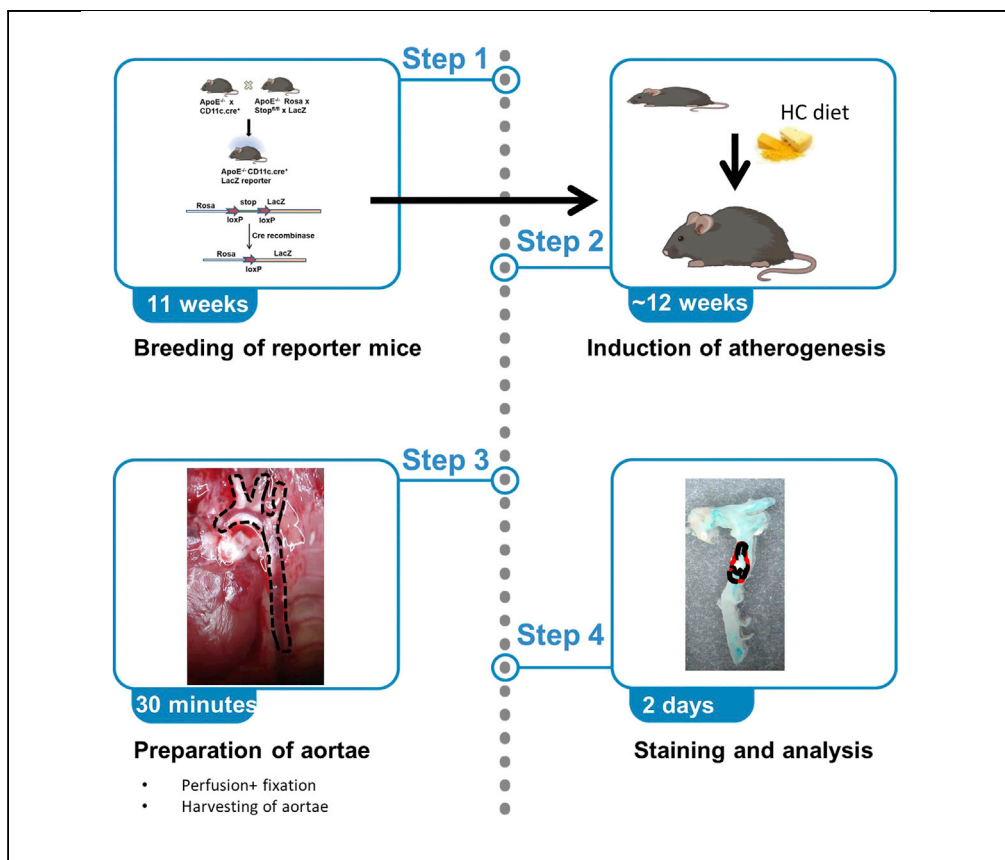


## Protocol

# Protocol to visualize CD11c<sup>+</sup> cells in atherosclerosis using LacZ reporter mice



Here, we describe an *in vivo* approach to visualize CD11c<sup>+</sup> cells in atherosclerosis. In particular, we use a protocol for X-Gal staining of immune cells within atherosclerotic plaques, which can be used as an alternative to analyze plaque composition and cell-specific molecules in atherogenesis. LacZ knockin mice have to be bred to mice carrying the CD11c<sup>Cre</sup> recombinase—both brought onto an ApoE<sup>-/-</sup> background—to be able to visualize this cell type of interest in the plaques by X-Gal staining. With this approach, different immune cells in atherogenesis can be examined.

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

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**Highlights**  
Detailed protocol to  
generate CD11c  
reporter mice via  
specific expression of  
LacZ

Detailed description  
of X-Gal staining in  
aortae of atheroscle-  
rotic CD11c-LacZ  
mice

Quantify LacZ-  
positive cells in  
atherosclerotic  
plaques

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## Protocol

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## SUMMARY

Here, we describe an *in vivo* approach to visualize CD11c<sup>+</sup> cells in atherosclerosis. In particular, we use a protocol for X-Gal staining of immune cells within atherosclerotic plaques, which can be used as an alternative to analyze plaque composition and cell-specific molecules in atherogenesis. LacZ knockin mice have to be bred to mice carrying the CD11c<sup>cre</sup> recombinase—both brought onto an ApoE<sup>-/-</sup> background—to be able to visualize this cell type of interest in the plaques by X-Gal staining. With this approach, different immune cells in atherogenesis can be examined.

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

## BEFORE YOU BEGIN

The protocol provided describes the specific steps for studying CD11c<sup>+</sup> cells and CD11c<sup>+</sup> derived ApoE in atherosclerosis. However, it can also be used for analysis of other immune cells such as macrophages.

So far, CD11c<sup>+</sup> cells have been detected in plaques using immunohistochemical or –fluorescent stainings. These stainings, however, are very difficult to achieve and hard to reproduce.

Animals: Ensure that all animal procedures were institutionally approved and comply with local ethics board guidelines. Experience with mouse handling is required.

Mice procured from a vendor should be acclimatized for at least 1–2 weeks prior to using them in the breeding program. Mice must be in a 12 h light/dark cycle and have access to food and water ad libitum. The present protocol was optimized for B6.129P2-Apoe<sup>tm1Unc/J</sup> (shortly: ApoE<sup>-/-</sup>) mice on C57BL/6J background. Testing of five mice per group is recommended as the minimum sample size.

## Institutional permissions

Experiments involving mice must conform to institutional and governmental guidance. The procedures in this protocol were approved by the Regierungspraesidium Tuebingen (approvals M03/10



and M16/15) and performed in accordance with the Guide for the Care and Use of Laboratory Animals and all other relevant ethical guidelines.

### Breeding of mice

⌚ Timing: 11 weeks

1. Prepare mutant mice expressing LacZ (B6.129S4-Gt(ROSA)26Sor<sup>tm1Sor</sup>/J; shortly: LacZ<sup>fl/fl</sup>) and breed them with ApoE<sup>-/-</sup> animals to get mice homozygous for ApoE knockout and heterozygously carrying the LacZ<sup>fl</sup> gene.
2. Simultaneously, prepare B6.Cg-Tg(ltgax-cre)1-1Reiz/J (shortly: CD11c<sup>cre</sup>) mice and breed them with ApoE<sup>-/-</sup> mice to get mice homozygous for ApoE knockout and carrying the CD11c<sup>cre</sup> gene heterozygously.
3. Crossbreed these two mouse-lines to get ApoE<sup>-/-</sup>CD11c<sup>cre/+</sup>LacZ<sup>fl/+</sup> mice. Mice not carrying the cre-recombinase serve as negative controls.

**Note:** Check the insertion of LacZ as well as Cre recombinase by genotyping – use the protocols provided by the mouse-providing companies (Jackson laboratory) and the primers listed in the [key resources table](#).

### Inducing atherogenesis

⌚ Timing: 6–12 weeks or longer, depending on study

4. Start feeding the mice with high cholesterol diet at the age of 6 weeks for different time periods.

**Note:** Carefully choose the type of animal diet – different diets come into question for different issues (e.g., Western diet vs. Paigen diet). Choose appropriate time points for evaluating different stages of atherogenesis.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Experimental models: Organisms/strains</b>		
ApoE <sup>-/-</sup> mice (Both male and female mice at the age of 6–8 weeks were used)	The Jackson Laboratory	Stock no. 002052; RRID: IMSR_JAX:002052
C57BL/6J mice (as controls. Both male and female mice at the age of 6–8 weeks were used.)	The Jackson Laboratory	Stock no. 000664; RRID: IMSR_JAX:000664
CD11c <sup>cre+</sup> mice (Both male and female mice at the age of 6–8 weeks were used.)	The Jackson Laboratory	Stock no. 008068; RRID: IMSR_JAX:008068
LacZ <sup>fl/fl</sup> mice (Both male and female mice at the age of 6–8 weeks were used.)	The Jackson Laboratory	Stock no. 003474; RRID: IMSR_JAX:003474
<b>Chemicals, peptides, and recombinant proteins</b>		
Ethanol	Carl Roth	Cat. no. T171.4
Formaldehyde solution 4%, buffered, pH 6.9	Merck	Cat. no. 1004960700
Glutaraldehyde	Merck	Cat. no. 354400
High cholesterol diet EF, 10 mm, Paigen	ssniff	Cat. no. S8127-E510
Invitrogen Agarose UltraPure™	Thermo Scientific	Cat. no. 16500500
Isoflurane	CP Pharma	Cat. no. 1214
Paraffine	Carl Roth	Cat. no. CN49.2
PBS without Ca and Mg	Gibco	Cat. no. 14190-169

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Roti-Histol	Carl Roth	Cat. no. 6640.1
X-Gal	Merck (Sigma-Aldrich)	Cat. no. 3117073001
<b>Oligonucleotides</b>		
ApoE (olMR0180)	Eurofins	5'-GCC TAG CCG AGG GAG AGC CG-3'
ApoE (olMR0181)	Eurofins	5'-TGT GAC TTG GGA GCT CTG CAG C-3'
ApoE (olMR0182)	Eurofins	5'-GCC GCC CCG ACT GCA TCT-3'
CD11c cre (olMR7841)	Eurofins	5'-ACT TGG CAG CTG TCT CCA AG-3'
CD11c cre (olMR7842)	Eurofins	5'-GCG AAC ATC TTC AGG TTC TG-3'
CD11c cre (olMR8744)	Eurofins	5'-CAA ATG TTG CTT GTC TGG TG-3'
CD11c cre (olMR8745)	Eurofins	5'-GTC AGT CGA GTG CAC AGT TT-3'
LacZ (BB01)	Eurofins	5'-CTC TGC TGC CTC CTG GCT TCT-3'
LacZ (BB02)	Eurofins	5'-CGA GGC GGA TCA CAA GCA ATA-3'
LacZ (RF127)	Eurofins	5'-GCG AAG AGT TTG TCC TCA ACC-3'
<b>Software</b>		
ImageJ	Wayne Rasband, NIH	<a href="https://imagej.nih.gov/ij/">https://imagej.nih.gov/ij/</a>

## MATERIALS AND EQUIPMENT

### Fixative solution for X-Gal staining

Reagent	Final concentration
Formaldehyde	2%
Glutaraldehyde	0.2%
K <sub>4</sub> F <sub>n</sub> (CN) <sub>6</sub>	2.5 mM
PBS pH 7.4	Fill up to 50 mL per mouse

△ **CRITICAL:** (Para-)Formaldehyde solution is a harmful material for humans. Handling (para-) formaldehyde solution should be done inside a chemical fume hood and wearing protective gears such as glasses, lab coat, and gloves.

### X-Gal stock solution

Reagent	Final concentration
X-Gal	40 mg/mL
Dimethylsulfoxide	10 mL

### X-Gal staining solution

Reagent	Final concentration
MgCl <sub>2</sub>	2 mM
K <sub>3</sub> F <sub>n</sub> (CN) <sub>6</sub>	2.5 mM
K <sub>4</sub> F <sub>n</sub> (CN) <sub>6</sub>	2.5 mM
PBS pH 7.4	Fill up to 500 mL

## STEP-BY-STEP METHOD DETAILS

### Preparing the mice for removal of the aortae

⌚ **Timing:** 30 min

These steps describe the preparation of the aortae to achieve a high quality X-Gal staining.

1. Euthanize the mice by placing them into a cage that is slowly filled with CO<sub>2</sub>. Wait until no more breathing could be observed. Go on with further procedure only after death has been confirmed reliably.

**Note:** Alternatively, mice could also be euthanized using inhaled anesthetics (Isoflurane, for example: Inhalation of 5.0 relative vol. % in an anesthesia chamber at an oxygen flow of 1 l/min) combined with blood deprivation or intraperitoneally injected anesthetics (Ketamine / Xylazine) combined with blood deprivation.

CAUTION: Use of anesthetic must be approved by the appropriate institutional authorities.

2. Cut the chest open and expose the chest cavity.
3. Perfuse the animal with 1 × PBS through the left ventricle until the liver appears to be whitish.
4. Fix the aortae in situ by slowly perfusing the mouse with 10 mL of the fixative solution. If a perfuser is used: perfuse at 60 mL/h for 10 min.

**Note:** The composition of the fixative solution depends on further processing: For X-Gal staining of whole mounts or cryosections the fixative contains 2% formaldehyde and 0.2% glutaraldehyde (in PBS) as this combination has proven to yield excellent fixation of a wide variety of tissues. However, the standard fixative for perfusion fixation, which is 4% paraformaldehyde in PBS, could also be used and we assume that this will not have a significant negative effect on the X-Gal staining outcome. Other fixatives like osmium tetroxide, mercuric chloride-based fixatives, precipitating fixatives (ethanol, methanol and acetone) or diimidazole fixation have not been used in our laboratory and are therefore not recommended using this protocol. The fixative solution can be stored at 4°C for a maximum of 4 weeks.

### Dissection and staining of the aorta

⌚ Timing: 2 days

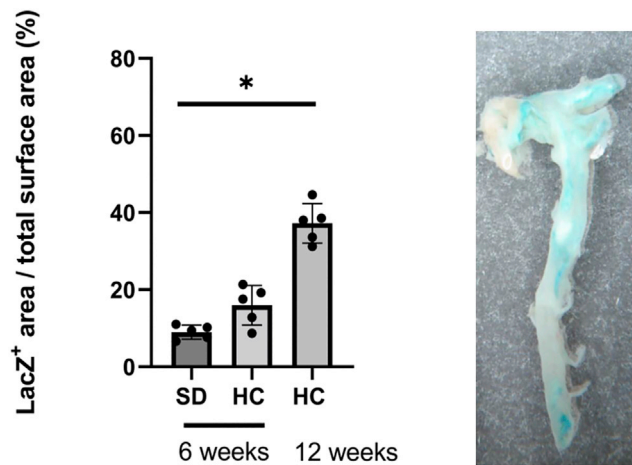
Here, we describe the process of the X-Gal staining and its quantification.

5. Remove internal organs as well as the lungs, trachea and esophagus. Only the heart with its attached vessels should be left now.
6. Cut ascending vessels, below the pharynx.
7. Grab the heart with forceps and lift it carefully to set the aorta under some tension.
8. Cut along the spine towards the tail.

**Note:** Avoid rupture of the aorta during dissection. It is very difficult to find its loose end in the body.

9. Transfer the excised aorta (still attached to the heart) into a 10-cm Petri dish with PBS and remove the heart by carefully cutting the left ventricle in 5 mm distance around the aortic valve.
10. Transfer aorta into a 1.5-mL microcentrifuge tube with 1 mL of fixative solution.
11. Postfix tissues for 30 min at RT with gentle shaking.
12. Wash fixed tissues three times for 5 min with PBS at RT with gentle shaking and process immediately for further analysis.
13. Incubate fixed aorta in 1.5 mL of X-Gal staining solution overnight in the dark at RT with gentle shaking.

**Note:** X-Gal stock solution (40 mg/mL) should be stored as 1 mL aliquots at –20°C. The staining solution should be stored in the dark at RT. Before use, X-Gal stock solution should be added to a final concentration of 1 mg/mL of X-Gal. Depending on the amount of tissue to be stained, 2–20 mL of X-Gal staining solution per mouse need to be prepared.



**Figure 1. Quantification of LacZ<sup>+</sup> area within plaque area**

Whole aortic area and blue area within the aorta is measured with an image analysis program like ImageJ. Here is an example of the accumulation of LacZ expressing CD11c<sup>+</sup> cells within the atherosclerotic lesions with time in high cholesterol fed ApoE<sup>-/-</sup> mice. Data are the mean +/- SD. \*p<0.05

14. Wash tissues three times for 5 min with PBS at RT with gentle shaking.
15. Take photos under a dissection microscope or stereo-microscope.
16. Measure LacZ positive (blue) area using ImageJ (see [Figure 1](#)).

**Note:** Optionally, aortae can be paraffin embedded, cut with a microtome and analyzed microscopically (see [Figure 2](#) and section “[quantification and statistical analysis](#)”). Then, quantification can also be performed with the paraffin sections using ImageJ (analysis of pixels per mm<sup>2</sup>). Co-stainings using immunohistochemistry can be performed to further characterize the LacZ positive cells.

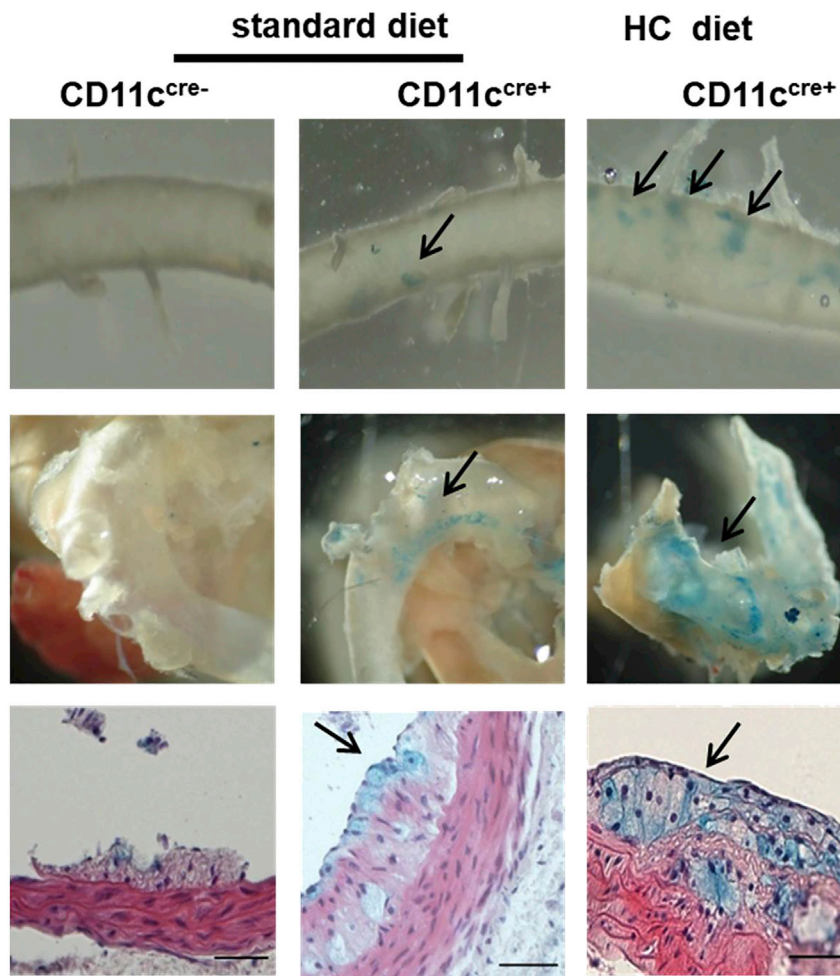
## EXPECTED OUTCOMES

This protocol helps to analyze different immune cells in the course of atherogenesis. CD11c<sup>+</sup> cells, for example, are very difficult to visualize within the plaques using established techniques such as immunohistochemistry or immunofluorescence. They could be quantified in aortae using FACS, however, it is not possible to see their distribution within the plaque. Using the “LacZ reporter mouse”, it is easily possible to localize and quantify their accumulation within the lesions ([Figures 1 and 2](#)). Using this protocol, we found that CD11c<sup>+</sup> cells accumulate within atherosclerotic plaques during the time course of atherogenesis in mice fed with high cholesterol diet ([Sauter et al., 2021](#)). In this study, we found these cells to be located mainly within the plaque-shoulders.

A major advantage of this model is, that there are also transgenic cre mice for other immune cells that play a role in atherosclerosis (for example, CD11b<sup>cre</sup>, F4/80<sup>cre</sup>). All of them can be mated to the LacZ<sup>fl/fl</sup> mouse on an ApoE<sup>-/-</sup> background and then visualized in atherosclerotic plaques via X-Gal staining.

To further characterize the LacZ positive cells, paraffin embedded sections (or alternatively cryo sections) could further be stained immunohistochemically, using specific antibodies. This provides the possibility to further determine the subtype of the LacZ positive CD11c<sup>+</sup> cells (for example by detecting CD45, CD86, CD11b, CD103).

The protocol can be modified to furthermore study “fate-mapping” of immune cells in atherogenesis. Therefore, bone marrow chimeras could be generated for example, using the LacZ reporter



**Figure 2.** Examples of X-Gal-stained aortae macroscopically as well as microscopically (scale bar: 50  $\mu$ m) containing plaques with LacZ expressing CD11c<sup>+</sup> cells

mice as bone marrow donors. With the help of “inducible cre mice” (Probst et al., 2003) you could even perform more in-depth analyses at different stages of the disease.

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Paraffine sections of X-Gal-stained tissue

⌚ Timing: 2 days

1. Dehydrate the tissue in 70, 80, and 95% ethanol, 45 min each, followed by three changes of absolute ethanol, 1 h each.
2. Clear tissue in Roti-Histol for 30 min. Exchange Roti-Histol and incubate tissue for another 15 min.
3. Isolate the region of interest for sectioning. Use a scalpel and trim tissue to the appropriate size for embedding in paraffin.
4. Immerse the tissue in paraffin, 3 $\times$  for 1 h to overnight.
5. Embed the tissue in paraffin. Place aorta in a small mold in appropriate orientation for cross-sectioning.

**Note:** Paraffin tissue blocks can be stored at RT for years.

6. Section the paraffin block at 6  $\mu\text{m}$  thickness on a microtome and let sections flatten by floating in a 40°C water bath containing distilled water.
7. Transfer sections onto glass slides suitable for immunohistochemistry (e.g., polylysine-coated slides). Allow slides to dry overnight at 37°C and store them at RT.
8. Deparaffinize and rehydrate sections. Place slides in a rack and perform the following washes: Roti-Histol, 2 $\times$  3 min; 100% ethanol, 2 $\times$  3 min; 95% ethanol, 1 $\times$  3 min; 70% ethanol, 1 $\times$  3 min; rinse slides with cold tap water.
9. If required, X-Gal-stained paraffin sections can be co-stained by conventional histochemistry (e.g., eosin, nuclear fast red) and/or immunohistochemistry.

### LIMITATIONS

Detecting CD11c<sup>+</sup> cells using the LacZ system is a very specific method and superior to classical immunostaining approaches. However, it has to be mentioned that the results obtained have to be interpreted carefully. With the cre recombinase system, all cells that ever expressed CD11c are targeted even if not actually showing CD11c on their surface – this could be circumvented by using inducible cre mice. It has also to be mentioned, that in some mouse strains “Cre mosaicism” is documented, resulting in incomplete recombination in all cells. Therefore, the protocol should ideally be supplemented with classical immunostaining methods.

### TROUBLESHOOTING

#### Problem 1

No or weak signal after X-gal staining (step 15).

#### Potential solution

Make sure that the fixative solution is prepared exactly according to this protocol. The fixation step is quite critical for detecting LacZ activity. Always compare your results to negative controls – we therefore use cre-negative litter mates. You can also consider using mice homozygously expressing LacZ.

#### Problem 2

Staining is not homogeneous or staining background is too prominent (step 15).

#### Potential solution

Use enough fixative/staining. Insufficient fixation/staining often might cause uneven staining.

The washings steps (steps 12 and 14) are very important, too – shortening the times of the washing steps will result in high “background staining”.

#### Problem 3

Blood cells or even a blood clot remaining within the vascular tree resulting in unspecific plaque staining (step 15).

#### Potential solution

Insufficient perfusion causes blood retention within the analyzed vessels. Make sure that the perfusion (steps 3 + 4) is performed rapidly and well before the formation of blood clots; this will instantly improve your results.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Harald F. Langer ([harald.langer@uksh.de](mailto:harald.langer@uksh.de)).

#### Materials availability

This protocol did not generate unique materials or reagents.



**Data and code availability**

This protocol did not generate unique materials or reagents.

**ACKNOWLEDGMENTS**

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**AUTHOR CONTRIBUTIONS**

M.S. and R.J.S. performed experiments, analyzed and compiled data, and wrote parts of the manuscript. M.T., S.F., and R.F. showed the methods, assisted with the experimental setup, and interpreted data. M.S., R.J.S., and H.F.L. interpreted data and wrote parts of the manuscript. H.F.L. conceived the project, analyzed data, and wrote the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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