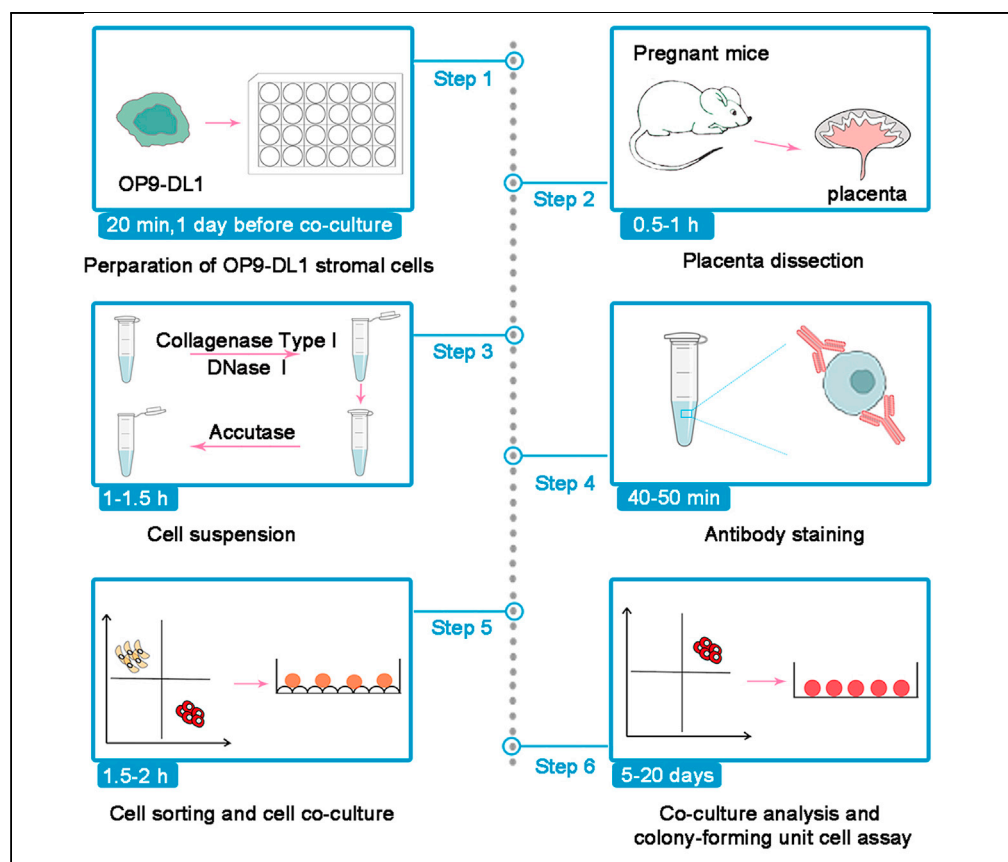


## Protocol

# Protocols for isolation and characterization of mouse placental hemogenic endothelial cells



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### Highlights

Dissection and digestion of the mouse placenta

Identification of placental hemogenic endothelial cells

Functional validation of placental hemogenic endothelial cell-derived cells

The murine mid-gestational placenta has been identified as a hematopoietic site during embryonic development. Here, we describe a protocol for isolation and characterization of the hemogenic endothelial (HE) cells from mouse placenta. We also describe techniques for dissection of placental tissues and for the optimization of tissue digestion and antibody conjugation conditions to identify HE cells via fluorescence-activated cell sorting.

## Protocol

# Protocols for isolation and characterization of mouse placental hemogenic endothelial cells

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## SUMMARY

The murine mid-gestational placenta has been identified as a hematopoietic site during embryonic development. Here, we describe a protocol for isolation and characterization of the hemogenic endothelial (HE) cells from mouse placenta. We also describe techniques for dissection of placental tissues and for the optimization of tissue digestion and antibody conjugation conditions to identify HE cells via fluorescence-activated cell sorting. For details on the usage and application of this protocol, please refer to Liang et al. (2021).

## BEFORE YOU BEGIN

⌚ Timing: 7–10 days

This protocol describes the steps to sort out HE cells from mouse placenta (Liang et al., 2021). It was primarily optimized using mid-gestational placenta, which was dissected from embryonic day (E) 11.0 pregnant mice. And the booking of the flow cytometer is carefully timed to match mouse breeding. Animal experiments and procedures have been approved by the Ethical Review Committee in the Institute of Zoology, Chinese Academy of Sciences.

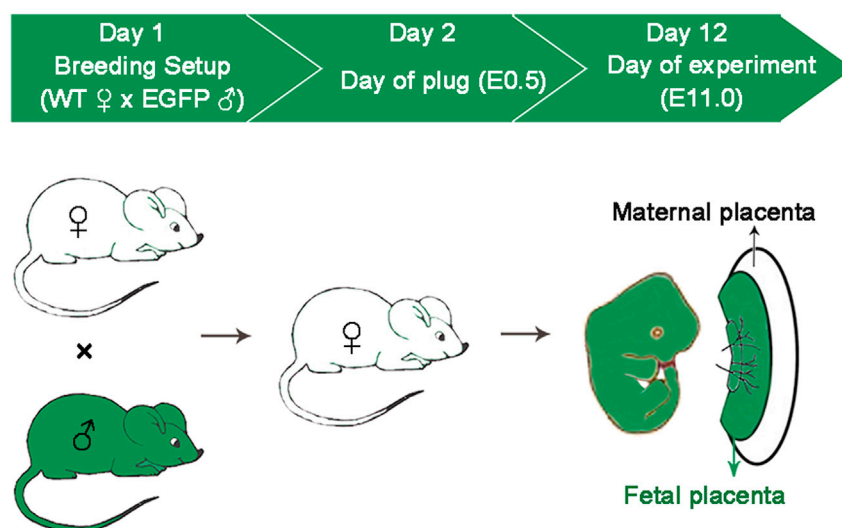
1. Order antibodies suitable for application. e.g., anti-CD31-PE-Cyanine 7, anti-CD45-APC-eFluor 780, anti-CD44-PE, anti-Ter119-PE-Cyanine 5 and 7-AAD.
2. Order hematopoietic cytokines for application. e.g., SCF, Flt3L and IL3.
3. Prepare Type I Collagenase: 1 mg/mL collagenase stock in 20% FBS in DPBS, filter using a 0.22  $\mu$ m filter, and store at  $-20^{\circ}\text{C}$ .
4. Order DNase I (2000 U/mL), Accutase, 1 $\times$ RBC lysis buffer, DMEM, MEM  $\alpha$  and DPBS.
5. Order 1 mL injection syringes, 20G and 25G needles.
6. Order 1.5 mL tubes, 60 mm dishes, 24-well plates and ultra-low attachment 24-well plates.

## Setup mouse mating for embryonic tissue

⌚ Timing: 10–12 days

7. Setup mating of mice
  - a. Move one male (age 8 weeks -12 months) to each of clean breeding cages;
  - b. Add one female (age 8–12 weeks) to each breeding cage in the afternoon.





**Figure 1. The strategy of breeding of mice and experimental timeline**

8. Check for vaginal plug the next morning, day of observation of vaginal plug is recorded as E0.5.
9. Wait the required amounts of days according to the embryonic day aimed to analysis.

**Note:** In our experiment, we have set up the EGFP homozygous or heterozygous male mice with wild type female mice for cell sorting (Figure 1A) (Alvarez-Silva et al., 2003).

### Preparation for the dissociation of placenta

⌚ Timing: 15 min

10. Prepare 1.5 mL tubes with digestion medium which is composed of 1 mL 1 mg/mL Collagenase Type I-A and add 4  $\mu$ L DNase I (2000U/mL).
11. Warm the digestion medium at 37°C.
12. Prepare 50 mL DMEM medium with 2% FBS (2% FBS/DMEM).
13. Prepare 60 mm dish with DPBS.

**Note:** All reagents should be prepared under sterile conditions.

### Preparation for OP9-dL1 stromal cells

⌚ Timing: 20 min

14. Prepare culture medium composed of MEM  $\alpha$ , 20% FBS, 1% PS and 1% LG;
15. Mouse OP9-DL1 stromal cells ( $2 \times 10^4$ – $4 \times 10^4$ ) are cultured in a 24-well plate for 1 day before co-culture.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
PE-Cyanine 5 anti-mouse Ter119 (Clone TER-119)	BioLegend	Cat# 116209
PE-Cyanine 7 anti-mouse CD31 (Clone 390)	eBioscience	Cat# 25-0311-82

(Continued on next page)

<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
APC-eFluor 780 anti-mouse CD45 (Clone 30-F11)	eBioscience	Cat# 47-0451-82
PE anti-Human/Mouse CD44 (Clone IM7)	eBioscience	Cat# 12-0441-81
7-amino-actinomycin D	BioLegend	Cat# 420404
PE-Cyanine 5 anti-mouse Ter119 (Clone TER-119)	BioLegend	Cat# 116209
<b>Chemicals, peptides, and recombinant proteins</b>		
Dulbecco's Phosphate Buffered Saline (DPBS)	Gibco	Cat# 14190144
Fetal bovine serum (FBS)	Gibco	Cat# 10099141C
Minimum Essential Medium $\alpha$ (MEM $\alpha$ )	Gibco	Cat# 12561056
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	Cat# 11995065
1 $\times$ Red Blood Cell (RBC) lysis buffer	eBioscience	Cat# 00-4333-57
MethoCult GF M3434	Stem Cell Technologies	Cat# 03434
Recombinant murine Flt3-Ligand	PeproTech	Cat# 250-31L
Recombinant murine SCF	PeproTech	Cat# 250-03
Recombinant murine IL3	PeproTech	Cat# 213-13
Collagenase Type I-A	Sigma	Cat# C2674
StemPro™ Accutase™	Gibco	Cat# A1110501
DNase I	NEB	Cat# M0303S
Penicillin-Streptomycin (PS)	Gibco	Cat# 15140122
L-Glutamine (LG)	Gibco	Cat# 25030081
<b>Experimental models: Cell lines</b>		
OP9-DL1	N/A	N/A
<b>Experimental models: Organisms/strains</b>		
Mouse: C57BL/6	SPF	N/A
Mouse: EGFP	SPF	N/A
<b>Others</b>		
ultra-low attachment 24-well plates	Corning	Cat# 3473
60 mm dish	Corning	Cat# 430166
1.5 mL tubes	Axygen	Cat# MCT-150-C
1 mL injection syringes	BD Pharmingen	Cat# 300841
20G and 25G needles	Kunming Huangbao Trading Co	Cat# ZSZT-6
24-well plate	Corning	Cat# 3524
cell strainers	BD Falcon	Cat# 352235
ophthalmic scissor	CAS Mart	Cat# YC-YL01001
0.22 $\mu$ m filter	Sartorius	Cat# 17597-K

## STEP-BY-STEP METHOD DETAILS

### Dissociation of mouse placenta

⌚ Timing: 0.5–1 h

This step describes the procedures required to remove the yolk sac, embryo proper and umbilical cord from a pregnant mouse, and to dissociate the placenta into a single-cell suspension. The dissection may be performed at room temperature, with sterile conditions.

1. Embryo proper, yolk sac and placenta are taken out of the uterus from a pregnant mouse.
2. Chose EGFP placentas by fluorescence microscopy.
3. Separate the placenta from embryo proper and yolk sac (Pereira et al., 2016).
4. Remove out maternal decidua attached placenta by inserting the two pairs of forceps between fetal and maternal sides and forcing them apart (Ottersbach and Dzierzak, 2009).
5. Placentas are placed in DPBS in a 60 mm dish on ice.

**Note:** Fresh placental tissue should be used to isolate HE cells for co-culture.

### Preparation of cell suspension

⌚ Timing: 1–1.5 h

This step is to disaggregate the placentas and prepare a single-cell suspension ready for flow cytometry analysis (Gekas et al., 2008).

6. Placentas are washed in DPBS twice on ice.

**Note:** To minimize contamination with maternal blood cells, the placenta is placed in a 60 mm dish and is rinsed several times with DPBS until no obvious blood clots observed. About 8–10 mL DPBS is needed to wash the blood clots of placentas.

7. Transfer per placenta into 1.5 mL tubes with pre-warmed digestion medium containing 1 mL 1 mg/mL Collagenase Type I-A that add 0.04% DNase I, followed by mechanical dissection with 20G and 25G needles.

**Note:** For each placenta at E11.0, prepare 1 mL Collagenase Type I-A, and a suitable volume should be adapted depending on the size of the placenta. Before dissecting per placenta with 20G and 25G needles, it is better to cut the placenta into fragments with ophthalmic scissor.

8. Digest placentas at 37°C for 30 min in a shaking incubator with 100 revolutions per minute.

9. Centrifuge tubes at 310 g for 5 min at 4°C and carefully discard the supernatant.

10. Placentas are washed in DPBS and centrifuged, and added 500 µL Accutase for 6–8 min at 37°C in a shaking incubator with 100 revolutions per minute.

11. Add 500 µL 2% FBS/DMEM and centrifuge tubes at 310 g for 5 min at 4°C and carefully discard the supernatant.

12. RBC lysis buffer (1 mL) treatment for 5–10 min at room temperature.

13. Then tissues are centrifuged and washed in 1 mL 2% FBS/DMEM.

14. Filter the cells using single-use cell strainers (BD Falcon, 352235) to make a single-cell suspension. Wash the strainer with 300 µL 2% FBS/DMEM into original tubes.

15. Centrifuge tubes at 310 g for 5 min at 4°C and prepare for antibody staining.

**Note:** If you used this protocol for other hematopoietic tissues, such as yolk sac or AGM region, the digestion steps, especially digestion time, may need to be optimized. Transfer per yolk sac or AGM tissue into 1.5 mL tubes with pre-warmed digestion medium containing 300 µL 1 mg/mL Collagenase Type I-A adding 1.2 µL DNase I, followed by mechanical dissection with 25G needles, and then digest it at 37°C for 15–20 minutes.

⚠ **CRITICAL:** It is important that all tissues should be digested. Make sure that there are no visible clumps of tissue after these steps.

### Stain with antibody for surface antigen

⌚ Timing: 40–50 min

This step is to label cells of single-cell suspension for surface antigens.

16. Prepare enough antibody dilution to stain all samples, according to antibody operating instruction. Prepare antibody dilution in 2% FBS/DMEM. For example, we dilute CD45-APC-eFluor 780 antibody into 1:100 for one test.

**Note:** Appropriate volume of antibody dilution should be prepared according to the concentration of cell. Cell number should be determined empirically, and can range from  $10^6$  to  $10^8$  cells/mL.

17. For each test, the cells are incubated with antibody mix for 30 min at 4°C, protected from light.

**Note:** For per 100  $\mu$ L antibody mix, it is composed of 1.25  $\mu$ L anti-CD44-PE, 0.25  $\mu$ L anti-CD45-APC-eFluor-780, 1.25  $\mu$ L anti-Ter119-PE-Cyanine 5, 2.5  $\mu$ L anti-CD31-PE-Cyanine 7 and 95  $\mu$ L 2% FBS/DMEM.

18. Centrifuge tubes at 310 g for 5 min at 4°C and carefully discard the supernatant.
19. Resuspend in appropriate volume of DPBS. And then the cells are incubated with 5  $\mu$ L 7-AAD (in 100  $\mu$ L DPBS) for 10 min at 4°C, protected from light.
20. Centrifuge tubes at 310 g for 5 min at 4°C and carefully discard the supernatant.
21. Resuspend in appropriate volume of 2% FBS/DMEM.

**△ CRITICAL:** For all samples, the volume of resuspended medium should depend on the cell number. Make sure the cell density is ranging from  $10^6$  to  $10^8$  cells/mL.

### Co-culture HE cells with OP9-DL1 cells

⌚ **Timing:** 1.5–2 h, depending on number of samples and cells

This step is to identify the hematopoietic potential of placental HE cells.

22. The HE cells (GFP<sup>+</sup>CD44<sup>+</sup>CD31<sup>+</sup>CD45<sup>+</sup>Ter119<sup>+</sup>) are sorted from the EGFP<sup>+</sup> placentas using a flow cytometer. The live cells are gated as 7-AAD-negative cells ([Figure 2A](#)).

**Note:** To eliminate the RBC, Ter119-negative cells are gated. To eliminate the contamination of maternal cells, GFP-positive cells are gated. Then the hematopoietic cells, CD45<sup>+</sup> cells were eliminated. HE cells are gated as GFP<sup>+</sup>CD44<sup>+</sup>CD31<sup>+</sup>CD45<sup>+</sup>Ter119<sup>+</sup> cells.

23. Change the OP9-DL1 culture medium to co-culture medium (500  $\mu$ L/well), which is composed of MEM  $\alpha$ , 10% FBS, 1% PS, 1% LG and cytokines, 100 ng/mL SCF, 100 ng/mL Flt3L and 100 ng/mL IL3 ([Li et al., 2013](#)).

**△ CRITICAL:** Fresh co-culture medium should be used.

24. Sort 100 HE cells to pre-prepared 24-well plate which has been cultured OP9-DL1 stromal cells 1 day before.

**Note:** The ratio of HE cells to OP9-DL1 stromal cells is 1: 200–400.

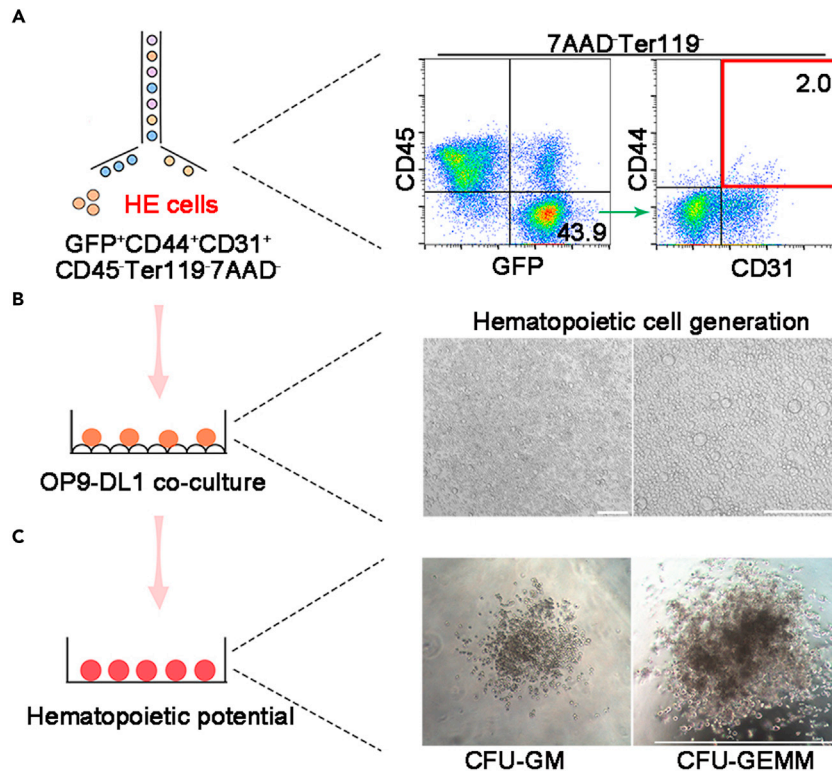
25. Put the 24-well plate into the incubator at 37°C, 5% CO<sub>2</sub>.

**Note:** Stop here and renew co-culture medium the next day.

### Colony-forming unit cell (CFU-C) assay

⌚ **Timing:** 12–20 days

This step is to detect the hematopoietic function of cells derived from HE cells.



**Figure 2. Experimental strategy and data example**

(Left) Experimental strategy for FACS analysis (A), co-culture analysis (B) and hematopoietic potential analysis (C). (Right) Representative experimental data. Scale bars, 50  $\mu$ m.

26. Renew half of the co-culture medium (250  $\mu$ L) every day.

**Note:** After 5 days, HE cells co-cultured with OP9-DL1 stromal cells are collected for further analysis (Figure 2B).

**△ CRITICAL:** Only remove the top of co-culture medium. Do not remove the entire medium, as this will lead to the loss of HE or hematopoietic cells.

27. Gently blow and collect all co-culture medium into 1.5 mL tubes and then centrifuge tubes at 310g for 5 min at 4°C.

**Note:** Hematopoietic cells are semi-adherent, and gently blowing of each well with a pipette gun can gain most of hematopoietic cells.

28. Filter the cells using single-use cell strainers to make a single cell suspension.

29. Incubate surface antibody (anti-CD45-APC-eFluor-780) for 30 min at 4°C, protected from light, as above mentioned.

30. Centrifuge tubes at 310 g for 5 min at 4°C and carefully discard the supernatant.

31. Resuspend in appropriate volume of DPBS, and then the cells are incubated with 7-AAD, protected from light, as above mentioned.

32. Centrifuge tubes at 310 g for 5 min at 4°C and carefully discard the supernatant.

33. Resuspend in appropriate volume of 2% FBS/DMEM.

34. The CD45<sup>+</sup> hematopoietic cells are harvested by FACS, and the live cells are gated as 7-AAD-negative cells.
35. Add 1000 collected cells into MethoCult GF M3434 medium (500  $\mu$ L) in ultra-low attachment 24-well plates for CFU-C assay.

**Note:** To avoid contamination, PS (5  $\mu$ L) should be selectively added into the medium. 1.0 mL injection syringe without a needle is used to mix the cells and medium.

36. After cultured for 7–15 days at 37°C in 5% CO<sub>2</sub>, the number of colonies, including CFU-granulocyte-monocyte (CFU-GM) and CFU-granulocyte, erythroid, macrophage, and megakaryocyte (CFU-GEMM) are distinguished based on the morphology and counted with an inverted microscope (Figure 2C).

## EXPECTED OUTCOMES

For a successful E11.0 placenta analysis, you should be able to analyze/sort on average 200–300 GFP positive HE cells. For a successful 100 HE cells co-culture analysis, you should be able to sort on average 500–1500 round hematopoietic cells and observe multiple hematopoietic colonies.

## LIMITATIONS

This protocol was optimized for placental tissue of mid-gestation. If used on parturient placental tissue, the enzymatic digestion time may be increased to fully digest all cells.

## TROUBLESHOOTING

### Problem 1

Low cell yields can have several causes:

- Loss of cells during the washing steps (step 10 and 14);
- Low concentration of antibody during the staining steps (step 17–19);
- High cell number of each sample during the antibody staining steps (step 17–19).

### Potential solution

- After centrifuging, the supernatant should be removed very gently;
- Properly increase the concentration of antibody;
- Decrease the cell number of each sample, and make sure the cell number is ranging from 10<sup>6</sup> to 10<sup>8</sup> cells/mL.

### Problem 2

Few hematopoietic cells can have several causes:

- Inappropriate cell density of OP9-DL1 stromal cells (step 15 in Before you begin section);
- Longtime to acquire HE cells (step 24);
- Loss of hematopoietic cells during changing culture medium (step 26).

### Potential solution

- 2×10<sup>4</sup>–4×10<sup>4</sup> OP9-DL1 stromal cells should be cultured in a 24-well plate for 1 day before co-culture;
- No more than 4 h to acquire HE cells;
- Only the top of co-culture medium should be removed very gently.



**Problem 3**

Few hematopoietic colonies can have several causes:

- Loss of cells during mixing the cells and MethoCult GF M3434 medium (step 35);
- Few hematopoietic cells are collected (step 34 and 35).

**Potential solution**

- Mix the cells and MethoCult GF M3434 medium with 1.0 mL injection syringe without a needle;
- Collect more than 1000 CD45<sup>+</sup> hematopoietic cells for CFU-C assay.

**Problem 4**

Incomplete digestion of placental tissue can have several causes:

- The contamination of maternal decidua (step 4);
- Insufficient digestion medium (step 7);
- Insufficient incubation time (step 8).

**Potential solution**

- Remove the maternal decidua between fetal and maternal sides with a microscope;
- Increase the volume of digestion medium to 1.5 mL for the large placenta;
- Mince the placentas into smaller fragments;
- Increase the incubation time, until no tissue should be visible after digestion. However, the incubation time could be no more than 50 min.

**Problem 5**

Cell contamination can have several causes:

- The contamination during placenta dissociation (step 1–5);
- The contamination during cell sorting (step 24).

**Potential solution**

- Make sure that all reagents are prepared under sterile conditions;
- Make sure that all experiments are performed under sterile conditions;
- Add suitable volume of PS when resuspend samples before FACS.

**RESOURCE AVAILABILITY****Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Feng Liu ([liuf@ioz.ac.cn](mailto:liuf@ioz.ac.cn)).

**Materials availability**

All the materials used in this protocol are commercially available.

**Data and code availability**

This study did not generate any unique data sets or code.

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### AUTHOR CONTRIBUTIONS

G.L. and F.L. designed the study and wrote the manuscript, with contributions from all authors. G.L. performed most of the experiments with the help from B.H. F.W. interpreted the data and assisted with the manuscript.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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