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Protocol
Detection of RNA-DNA hybrids by immunostaining in meiotic nuclei of Saccharomyces cerevisiae

Xuan Yang¹,⁴ and Liangran Zhang¹,²,³,⁵,*

¹Center for Reproductive Medicine, Cheeloo College of Medicine, State Key Laboratory of Microbial Technology, Shandong University, Jinan, Shandong 250012, China
²Advanced Medical Research Institute, Shandong University, Jinan, Shandong 250012, China
³Shandong Provincial Key Laboratory of Animal Resistance Biology, College of Life Sciences, Shandong Normal University, Jinan, Shandong 250014, China
⁴Technical contact
⁵Lead contact
*Correspondence: zhangliangran@sdu.edu.cn
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SUMMARY
RNA transcripts can anneal with template DNA strands to form RNA-DNA hybrids (or R-loops if the non-template DNA strands exist), which play a variety of roles in many physiological processes. Here, we provide an accessible and reproducible approach for immunofluorescent staining of RNA-DNA hybrids with the S9.6 antibody in spread meiotic nuclei of Saccharomyces cerevisiae. This protocol allows the examination of RNA-DNA hybrids as clearly distinguishable foci and the co-localizations of RNA-DNA hybrids with other proteins.

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

BEFORE YOU BEGIN
This method was used in a recent publication (Yang et al., 2021) to explore the role of RNA-DNA hybrids during the meiosis in Saccharomyces cerevisiae. The specificity of the S9.6 antibody was also evaluated. This method should be suitable for detecting RNA-DNA hybrids in all types of spread cells. However, due to the limited sensitivity of immunostaining assay, cells with few RNA-DNA hybrids may be difficult to be detected. This protocol was illustrated with samples prepared from the rnh1Δrnh201Δhpr1Δ mutant which has a high level of RNA-DNA hybrids. This protocol can also be used to simultaneously detect other target proteins by immunostaining and thus also their colocalization.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse monoclonal anti-RNA-DNA hybrid [S9.6] (1:1,000 dilution) | Kerafast | Cat # ENH001 RRID: AB_2687463 |
| Donkey anti-Mouse Alexa 488 Antibody (1:1,000 dilution) | Thermo Fisher Scientific | Cat # A-21202 RRID: AB_141607 |
| Chemicals, peptides, and recombinant proteins |        |            |
| Yeast extract       | BD Bioscience | Cat # 212720 |
| Bacto Peptone        | BD Bioscience | Cat # 211677 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Yeast nitrogen base without amino acids | BD Bioscience | Cat # 291920 |
| Potassium acetate | Sigma-Aldrich | Cat # V900213 |
| Potassium phthalate monobasic | Sigma-Aldrich | Cat # V900012 |
| Potassium hydroxide | Sigma-Aldrich | Cat # 221473 |
| D-(-)-Raffinose pentahydrate | Sigma-Aldrich | Cat # R0250 |
| Glycerol | Sigma-Aldrich | Cat # V900122 |
| Agar | Solarbio | Cat # A8190 |
| Antifoam 204 | Sigma-Aldrich | Cat # A8311 |
| D-Sorbitol | Sigma-Aldrich | Cat # S1876 |
| DAPI | Sigma-Aldrich | Cat # D9542 |
| D-(-)-Glucose | Sigma-Aldrich | Cat # G8270 |
| DL-Dithiothreitol | Sigma-Aldrich | Cat # V900830 |
| Potassium chloride | Sigma-Aldrich | Cat # V900068 |
| Sodium chloride | Sigma-Aldrich | Cat # V900058 |
| Sodium hydroxide | Sigma-Aldrich | Cat # S5881 |
| Zymolyase 100T | nacalai tesque | Cat # M7H7986 |
| MES hydrate | Sigma-Aldrich | Cat # M2933 |
| Paraformaldehyde | Sigma-Aldrich | Cat # P6148 |
| Bovine Serum Albumin | Sigma-Aldrich | Cat # A1933 |
| Photo-Flo 200 | Kodak | Cat # 146-4510 |
| Trizma Base | Sigma-Aldrich | Cat # V900483 |
| Ethylenediaminetetraacetic acid disodium salt dihydrate | Sigma-Aldrich | Cat # V900081 |
| Magnesium chloride hexahydrate | Sigma-Aldrich | Cat # V900020 |
| HCl | Sinopharm | Cat # 7647-01-1 |
| Mounting Medium with DAPI-Aqueous, Fluoroshield | Abcam | Cat # ab104139 |
| Sucrose | Sigma-Aldrich | Cat # V900116 |
| RNase H | New England Biolabs | Cat # M0297L |

**Experimental models: Organisms/strains**

| Organism | Strain | Notes |
|----------|--------|-------|
| *S. cerevisiae* (SK1 background) | Strain LZY819: ZIP3-13myc::Hygromycin B/**, URA3::CYC1p-LacI-GFP/**, scp1(Ch XV telomere)::LacO-LEU2/** | Yang et al. (2021) N/A |
| *S. cerevisiae* (SK1 background) | Strain LZY2919: ZIP3-13myc::Hygromycin B/**, URA3::CYC1p-LacI-GFP/**, scp1(Ch XV telomere)::LacO-LEU2/**, mh1::kanMX6/*, mh2014::natNT2/*, hpr1::kanMX6/* | Yang et al. (2021) N/A |

**Software and algorithms**

| Software | Source | Website |
|----------|--------|---------|
| NanoDrop 2000c | Thermo Scientific | https://www.thermofisher.cn/cn/zh/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy |
| ImageJ | Schneider et al., 2012 | https://imagej.nih.gov/ij/ |

**Other**

| Equipment | Source | Identifier |
|-----------|--------|------------|
| 175 mL PP Centrifuge Tube | Falcon | Cat # 352076 |
| 15 mL centrifuge tubes | Sigma-Aldrich | Cat # CLS430791 |
| 10 mL Pipette | NEST Scientific | Cat # 327001 |
| Disposable Cuvettes | Thermo Fisher Scientific | Cat # T-7H114955129 |
| Microscope slides | CITOTEST | Cat # 1AS105 |
| 24 x 50 mm microscope cover glass | CITOTEST | Cat # 10212450C |
| 24 x 60 mm Microscope cover glass | CITOTEST | Cat # 10212460C |
| 15 x 150 mm Test tube | N/A | N/A |
| 2 L flask | N/A | N/A |
| Clear nail polish | N/A | N/A |
| Fluorescence microscope | Zeiss | AxioImager.Z2 |
## MATERIALS AND EQUIPMENT

### YPG plates

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Yeast Extract            | 1%                  | 10 g     |
| Bacto Peptone            | 1%                  | 20 g     |
| D-(-)-Glucose (40%)      | 2%                  | 50 mL    |
| Agar                     | 2%                  | 20 g     |
| ddH₂O                    | n/a                 | Up to 1 L|
| Total                    |                     | 1 L      |

**Note:** Sterilize by autoclaving and pour the medium into plates while it is still warm. After the medium has solidified, store at 4°C for up to 4 weeks.

### YPD plates

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Yeast Extract            | 1%                  | 10 g     |
| Bacto Peptone            | 1%                  | 20 g     |
| D-(-)-Glucose (40%)      | 2%                  | 50 mL    |
| Agar                     | 2%                  | 20 g     |
| ddH₂O                    | n/a                 | Up to 1 L|
| Total                    |                     | 1 L      |

**Note:** Sterilize by autoclaving and pour the medium into plates while it is still warm. After the medium has solidified, store at 4°C for up to 4 weeks.

### YPD medium

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Yeast Extract            | 1%                  | 10 g     |
| Bacto Peptone            | 1%                  | 20 g     |
| D-(-)-Glucose (40%)      | 2%                  | 50 mL    |
| ddH₂O                    | n/a                 | Up to 1 L|
| Total                    |                     | 1 L      |

**Note:** Sterilize by autoclaving and store at 4°C for up to 4 weeks.

### SPS II medium

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Yeast Extract            | 0.5%                | 5 g      |
| Bacto Peptone            | 1%                  | 10 g     |
| Yeast nitrogen base without amino acids | 0.17% | 6.7 g |
| Potassium acetate        | 1%                  | 10 g     |
| Potassium phthalate monobasic | 0.05 M | 10.2 g |
| ddH₂O                    | n/a                 | Up to 1 L|
| Total                    |                     | 1 L      |

**Note:** Adjust the pH value to 5.5 with 10 M KOH. Sterilize by autoclaving, then add two drops of antifoam, mix at least 0.5 h, and store at 25°C for no more than 1 week.
Note: Sterilize by autoclaving, then add two drops of antifoam, mix at least 0.5 h, and store at 25°C for no more than 1 week.

### ZK buffer

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| Tris-HCl pH 7.5 (1 M)    | 50 mM               | 2.5 mL  |
| KCl (3 M)                | 0.5 M               | 8.3 mL  |
| ddH₂O                    | n/a                 | 39.2 mL |
| Total                    | n/a                 | 50 mL   |

Note: Store at 25°C.

### Zymolyase 100T solution

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| Zymolyase 100T           | 20 mg/mL            | 30 mg   |
| D-(-)-Glucose (40%)      | 2%                  | 75 μL   |
| Tris-HCl pH 7.5 (1 M)    | 50 mM               | 75 μL   |
| ddH₂O                    | n/a                 | Up to 1.5 mL |
| Total                    | n/a                 | 1.5 mL  |

Note: Store at −20°C.

### MES Wash buffer

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| D-Sorbitol (1 M)         | 1 M                 | 182.2 g |
| MES hydrate              | 0.1 M               | 19.6 g  |
| EDTA (0.5 M)             | 1 mM                | 2 mL    |
| MgCl₂ (1 M)              | 0.5 mM              | 500 μL  |
| ddH₂O                    | n/a                 | Up to 1 L |
| Total                    | n/a                 | 1 L     |

Note: Once all the powder dissolved completely, add 1 M NaOH dropwise to adjust the pH to 6.5. Sterilize by filtration and store at 4°C for up to 4 weeks.

### 3% paraformaldehyde with 3.4% sucrose

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| Paraformaldehyde         | 3%                  | 0.75 g  |
| Sucrose                  | 3.4%                | 0.85 g  |
| ddH₂O                    | n/a                 | Up to 25 mL |
| Total                    | n/a                 | 25 mL   |
Note: Dissolve paraformaldehyde in ~60°C warm ddH₂O with several drops of 1 M NaOH, adjust the pH to 6.5 with several drops of 1 M HCl. Sterilize by filtration and store at 4°C for up to 4 weeks.

△ CRITICAL: Paraformaldehyde is on the hazardous substance list. Harmful by inhalation or swallowing. Irritates eyes, respiratory system, and skin. Maybe sensitized by contact with skin, a suspected carcinogen. Please handle with caution and wear gloves, lab coats, and necessary protection to avoid direct body contact.

Note: Store at 25°C.

## TBS

| Reagent          | Final concentration | Amount     |
|------------------|---------------------|------------|
| Tris-HCl pH 8.0  (1 M) | 25 mM               | 10 mL      |
| NaCl (5 M)       | 136 mM              | 10.88 mL   |
| KCl (3 M)        | 3 mM                | 400 µL     |
| ddH₂O            | n/a                 | Up to 400 mL |
| Total            | n/a                 | 400 mL     |

Note: Store at 25°C.

## STEP-BY-STEP METHOD DETAILS

This method was modified from Koszul et al. (2009), Grubb et al. (2015), Börner and Cha (2015a), and Börner and Cha (2015b).

### Meiotic synchronization

© Timing: 6 days

Upon starvation for nitrogen in the presence of a nonfermentable carbon source, *S. cerevisiae* diploid cells will do meiosis. Therefore, media with less nutrients are applied to arrest yeast cells in G1 phase and then promote meiosis more synchronously and efficiently (Elrod et al., 2009).

1. Patch Cells from glycerol storage at ~80°C onto YPG plates and incubate at 30°C overnight (~14–16 h).

   Note: Glycerol is a non-fermented carbon source. Yeast strains with mitochondrial defects can be screened out on YPG plates. In order to prevent yeast cells entering meiosis in advance, usually do not let yeast cells grow on YPG for more than 16 h.

2. Streak yeast cells onto YPD plates and incubate at 30°C for 2–3 days (54–56 h) to obtain single colonies.

3. Inoculate a whole colony into 4 mL YPD liquid medium in a test tube. Grow at 30°C for 24 h on a roller. Set up three cultures for each strain.

4. Pick two good YPD cultures for each strain (Figure 1). Dilute each one into 150 mL SPS II medium in a 2 L flask: 1/500 (300 µL) for WT strain, and 1/50 (3 mL) for *rnh1Δ rnh201Δ hpr1Δ* mutant, incubate at 30°C for ~16 h in a shaker with 200 rpm. Troubleshooting 1.

   Note: After 24 h in YPD medium, a good culture gives enough sediment at the tube bottom, meanwhile, the liquid looks slightly cloudy (tube 1). It should also be taken into account that some mutants grow slowly and thus have few sediment (tube 2). It is worth noting that if all cells settle down to the tube bottom with very clear liquid on the top, you should check...
whether it is a haploid yeast (tube 3). In contrast, if the culture is too cloudy with little sediment at the bottom, the culture may be contaminated (tube 4).

5. For each strain, select one better culture to prepare the meiosis culture.
   a. Three steps to determine the better culture.
      i. Optical density. The OD600 of the undiluted culture measured with a spectrophotometer usually is 2.7–2.8, which corresponds to ~1 × 10^8 cells per mL. Use SPS II medium as a blank control.
      ii. Percentage of G1/G0 cells. By DAPI staining, assess the percentage of cells in vegetative S-phase (small buds without DAPI bodies) and mitosis (large buds with DAPI at or stretched across the neck) (Figure 2). These cells should be < 10%. The absence of such mitotic figures may indicate the culture has prematurely entered meiosis.
      iii. Cell Morphology. 4–8 daughter and mother cells at similar size are beaded (Figure 3).

   Note: Determine the synchronized culture as quickly as possible.

   b. Collect cells by centrifugation in 175 mL centrifuge bottles at 1,800 × g for 2 min, 25°C.
   c. Resuspend cells with 150 mL prewarmed SPM.
   d. Repeat steps b and c.
   e. Transfer each resuspended culture into one 2 L flask.
   f. Incubate cultures in a shaker incubator at 30°C, ~200 rpm. Record the time as 0 h.

   Note: To ensure a better meiosis time-course, prewarm all media used in step 5°C–30°C in an incubator before use.

6. Collect a proper amount of samples at desired time points according to your experimental purpose. For chromosome spreading, collect 10 mL cultures every hour during 0–8 h in SPM to detect RNA-DNA hybrids by immunostaining.
Note: Let the samples sit on ice before you use it. And the differences between WT and mutants in meiosis progression should be taken into account when you decide the time point to collect samples according to your experimental purpose.

**Chromosome spreading**

© Timing: 1–2 h

In the following steps, spheroplasts are prepared and nuclei are spread on slides. Surface spreading of yeast nuclei results in expansion of chromatin with minimal loss of bound proteins (Grubb et al., 2015).

7. Collect 10 mL of meiotic culture with a 15 mL centrifuge tube, and pellet at 1,000 × g for 2 min, 25°C.
8. Discard the supernatant and resuspend cells with 2.5 mL 200 mM Tris-HCl (pH 7.5).
9. Add 50 μL 1 M DTT and mix well.
10. Let stand at 25°C for 2 min.
11. Pellet at 1,000 × g for 2 min, 25°C.
12. Discard the supernatant and resuspend with 2.5 mL ZK buffer.
13. Add 5 μL zymolyase 100T solution and mix well. Incubate at 30°C on a roller for ~25 min, resuspend cells by inverting tubes every 5–10 min during incubation. Troubleshooting 2.

Note: Check progress by placing ~1 μL of mixture into a large drop of water on a slide and observe under a light microscope. If >90% of cells burst within 1 min, the spheroplasts are ready, then put the samples on ice to stop the reaction.

14. Pellet cells at 800 × g for 2 min.
15. Discard the supernatant and resuspend in 5 mL cold MES wash buffer.
16. Pellet cells at 800 × g for 2 min.
17. Discard the supernatant and resuspend cells in a suitable volume of MES wash buffer (can just barely see through suspension if you lift it up against the light). Troubleshooting 3.

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**Figure 2. Examples of DAPI staining in step 5.a.ii**

(A) A cell (no bud) in vegetative G1/G0-phase.
(B) A cell with a small bud (no DAPI signal in the bud or at the neck) in vegetative S-phase.
(C) A cell with a large bud (with DAPI at or stretched across the neck) in mitosis. Scale bar, 5 μm.
18. Put 20 µL cells on a slide.
19. Add 40 µL 3% paraformaldehyde with 3.4% sucrose to cells, then quickly add 80 µL 1% lipsol. Mix well with swirling.
20. Check under a microscope, when ~90% of cells explode (usually within 1 min). Then add 80 µL 3% paraformaldehyde with 3.4% sucrose to the mixture and mix.
21. Spread with a clean glass pipet. Troubleshooting 4.
22. Dry in air. It may take more than 4 h.
23. Store at −20°C in a slide storage box for 2–3 months, or at −80°C indefinitely.

**Immunofluorescence staining**

© Timing: 13–14 h

24. Dip slides vertically in 0.2% photoflo for 30 s.
25. Allow to dry.
26. Dip in 1× TBS in a horizontal Coplin jar for 15 min.
27. Drain the liquid thoroughly but do not dry it.
28. Place the slides horizontally in a moist chamber. Add 200 µL 1× TBS with 1% BSA, cover with a 24 × 60 mm coverslip and incubate for 10 min at 25°C.
29. Drain as above.
30. Dilute the S9.6 antibody using 1× TBS with 1% BSA (1:1,000 dilution works well). Apply 200 µL diluted antibody solution to each slide.
31. Cover with a coverslip and incubate overnight (~8–10 h) at 4°C in a moist chamber.
32. Shake the slide gently to remove the coverslip.
33. Apply 1×TBS to cover the slide (~700 µL) and incubate for 5 min at 25°C.
34. Drain.
35. Repeat steps #33–34 twice.
36. Add 200 µL diluted secondary antibody (1:1,000 of donkey anti-Mouse Alexa 488 antibody in 1× TBS with 1% BSA) to each slide under subdued light.
37. Cover with coverslip and incubate 1–3 h in a moist chamber in the dark at 25°C.
38. Remove coverslip and wash with 1× TBS as before.
39. Dry in the dark. Put 1 drop of antifade with DAPI. Cover with 24 × 50 mm coverslip, and seal with clear nail polish.
40. Store at −20°C in a 100-slide storage box for 1–2 months, or store at −80°C indefinitely.
**S9.6 antibody specificity test**

© Timing: 16–17 h

The S9.6 antibody has a highly specific affinity for RNA-DNA hybrids, but it may also bind to other AT-rich RNA-RNA strands, especially the abundant rRNA, although with a much lower affinity (Harnton et al., 2018; Smolka et al., 2021). If necessary, use a RNase H treated sample as a negative control to evaluate the non-specific background. RNase H is an endoribonuclease that specifically hydrolyzes the RNA strands in RNA-DNA hybrids (Lockhart et al., 2019). Given the instability of single-stranded RNA in vitro, the interference of rRNA on RNA-DNA staining results would be small. If necessary, RNase A in 300 mM NaCl can be used to minimize the effects of rRNA. Under this condition, RNase A can digest RNA but not that in RNA-DNA hybrids (Halász et al., 2017).

41. Use a slide that you know the signal of RNA-DNA-hybrids can be detected. Dip the slide into 0.2% photoflo for 30 s.
42. Air dry.
43. If necessary, test the specificity of S9.6 antibody.
   a. For the use of RNase H, dilute the RNase H enzymes using 1 × RNase H buffer (1:200 works well). Add 200 μL diluted RNase H enzymes to each slide.
   b. For the use of RNase A, dilute the RNase A (10 mg/mL) to 0.6 ng/μL with 300 mM NaCl solution. Add 200 μL diluted RNase A to each slide.
44. Cover with coverslip and incubate in moist chamber for 2–3 h at 37°C.
45. Remove coverslip and wash in TBS as before.
46. Perform S9.6 immunofluorescence staining according to steps 28–40.

**Fluorescence microscopy**

© Timing: 1–3 h

47. Take images with a 100× 1.6 NA oil-immersion objective on two channels individually to observe the signal of DAPI and RNA-DNA hybrids.

**EXPECTED OUTCOMES**

This protocol provides a simple way to detect RNA-DNA hybrids. Expected outcomes are demonstrated in Figure 4. Troubleshooting 5.

**LIMITATIONS**

Detection of RNA-DNA hybrids by immunostaining with the S9.6 antibody is simple and powerful. This method has two limitations: (1) immunostaining usually has a high detection limitation than other methods such as sequencing, and (2) the appearance of hybrid foci may depend on the quality of spreads, e.g., clustered signal may appear in underspread nuclei (the diameter of spread nuclei <5 μm) and only weak signal may be detected in overspread nuclei.

**TROUBLESHOOTING**

**Problem 1**
A low cell density in SPS II medium after ~16 h incubation (See step 4).

**Potential solution**
First check to confirm the SPS II medium is prepared correctly, especially the pH is 5.5. Inoculate more YPD culture into SPS II medium in step 4.
Problem 2
The appropriate diameter of spread nuclei is 6–7 μm. The nucleus may be underspread (<5 μm) or overspread (>8 μm).

Potential solution
This problem is most likely due to cells being less or over digested with zymolyase. If nuclei are underspread, use more zymolyase solution and/or a longer incubation time. If nuclei are overspread, use less zymolyase solution and/or a shorter incubation time. (See step 13).

Problem 3
Few nuclei are observed under a fluorescence microscope.

Potential solution
Suspend cells with less MES wash buffer (See step 17).

Problem 4
Many nuclei are overstretched or looks more compacted (Figure 5).

Figure 4. Detection of RNA-DNA hybrids in meiotic nuclei
Spread meiotic nuclei are immunostained with the S9.6 antibody to detect RNA-DNA hybrids (Green). Nuclei are stained with DAPI (Blue). Representative images show RNA-DNA hybrids in surface spread meiotic nuclei from WT and the mhl1Δmnh201Δhpr1Δ mutant (RNase H or RNase A pretreated samples are used as controls). Scale bar, 5 μm.
Potential solution
When spread nuclei, make sure the glass pipet only touch the liquid but not the slide to avoid stretching nuclei (See step 21). If there are many “compact” nuclei, this is usually because cells are not well digested. A higher concentration of paraformaldehyde or other fixative, e.g., methanol, can also cause nuclei hard to be fully spread.

Problem 5
Poor signal/noise ratio or high background.

Potential solution
Try one or the combination of several suggestions. Overnight incubation (~8–10 h) with a lower concentration of primary antibody at 4°C is highly recommended. Increasing blocking time with a higher concentration of BSA (e.g., 5%); increasing wash times after incubation with the primary and secondary antibodies (See steps 30, 31, 33, and 38).

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Liangran Zhang. (zhangliangran@sdu.edu.cn).

Materials availability
All materials associated with this study and the original research study (Yang et al., 2021) are available upon request to the lead contact, Liangran Zhang. (zhangliangran@sdu.edu.cn).

Data and code availability
This study did not generate any new datasets or code.

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AUTHOR CONTRIBUTIONS
X.Y. and L.Z. wrote the manuscript. X.Y. performed the experiments. L.Z. supervised the study.

DECLARATION OF INTERESTS
The authors declare no competing interests.
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