Generation and assessment of high-quality mouse oocytes and embryos following nicotinamide mononucleotide administration

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Protocol
Generation and assessment of high-quality mouse oocytes and embryos following nicotinamide mononucleotide administration

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SUMMARY
The low quality of oocytes is one of the main causes of the suboptimal reproductive outcome of female mammals with advanced maternal age. Here, we present a detailed protocol to obtain high-quality oocytes and embryos from aged mice by nicotinamide mononucleotide (NMN) administration. We also describe fluorescence staining procedures to assess the organelle dynamics in oocytes, and in vitro fertilization and embryo culture systems to evaluate the influence of NMN on the fertilization ability and embryonic development potential. For complete information on the use and execution of this protocol, please refer to Miao et al. (2020).

BEFORE YOU BEGIN
Preparation of NMN solution

© Timing: 10 min

1. Dissolve NMN in sterile PBS to prepare the solution at concentration of 100 mg/mL.

NMN administration and oocyte superovulation

© Timing: 10 days

2. Intraperitoneally inject 14–16-month-old ICR female mice with 0.1 mL of PBS (control) or 100 mg/mL NMN (200 mg/kg body weight per day) using a 1 mL syringe with 20G needle for 10 consecutive days.

△ CRITICAL: The administration dose should be optimized by gradient test (0–1,000 mg/kg body weight) according to the age and strain of the mice. (See Troubleshooting 1)

3. At the 8th day, intraperitoneally inject female mice with 10 IU pregnant mare serum gonadotropin (PMSG).
4. After 44–48 h, intraperitoneally inject female mice with 10 IU human chorionic gonadotropin (hCG).
**Note:** 5 IU of PMSG and hCG are usually injected for superovulation in young mice. Aged mice are not sensitive to the gonadotrophins, and thus injection dose may need to be increased to 10 IU when necessary. (See Troubleshooting 2)

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse monoclonal anti-α-tubulin-FITC | Sigma-Aldrich | Cat# F2168, RRID: AB_2827403 |
| Chemicals, peptides, and recombinant proteins |        |            |
| Nicotinamide mononucleotide | Sigma-Aldrich | Cat# N3501 |
| M2 medium | Sigma-Aldrich | Cat# M7167 |
| M16 medium | Sigma-Aldrich | Cat# M7292 |
| Albumin from bovine serum | Sigma-Aldrich | Cat# A1933 |
| Mineral oil | Sigma-Aldrich | Cat# M8410 |
| Triton X-100 | Sigma-Aldrich | Cat# V900502 |
| Tween 20 | Sigma-Aldrich | Cat# V900548 |
| Tyrode’s solution, acidic | Sigma-Aldrich | Cat# T1788 |
| Lens Culinaris Agglutinin (LCA)-FITC | Thermo Fisher Scientific | Cat# L32475 |
| Phosphate-buffered saline | Thermo Fisher Scientific | Cat# 20012027 |
| Hoechst 33342 | Thermo Fisher Scientific | Cat# H3570 |
| Propidium iodide | Thermo Fisher Scientific | Cat# P3566 |
| EmbryoMax Human Tubal Fluid (HTF) medium | Millipore | Cat# MR-070-D |
| EmbryoMax KSOM medium | Millipore | Cat# MR-106-D |
| 4% paraformaldehyde | Santa Cruz Biotechnology | Cat# 281692 |
| Pregnant mare’s serum gonadotropin (PMSG) | Janchun (Nanjing, China) | Cat# A006 |
| Human chorionic gonadotropin (HCG) | Janchun (Nanjing, China) | Cat# A001-2 |
| Critical commercial assays |        |            |
| MitoTracker Red CMXRos | Thermo Fisher Scientific | Cat# M7512 |
| Annexin V-FITC Apoptosis Detection Kit | Beyotime (Hangzhou, China) | Cat# C1062 |
| Reactive Oxygen Species Detection Kit | Jancheng (Nanjing, China) | Cat# E004 |
| Experimental models: organisms/strains |        |            |
| ICR mice | Animal Core Facility of Nanjing Medical University | N/A |
| Other |        |            |
| Stereo microscope | Olympus | SZ-61 |
| CO₂ incubator | Thermo Fisher Scientific | HERAcell 150 |
| Heating stage | CBL Photoelectron Technology | Model 100 |
| Fine-tip forceps | Rhino | SW14 |
| Glass slide | Citoglas | Cat# 7105P |
| 35 mm Petri dish | Thermo Fisher Scientific | Cat# 121V |
| ProLong Gold antifade mountant | Thermo Fisher Scientific | Cat# P10144 |

### MATERIALS AND EQUIPMENT

#### NMN solution

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| NMN     | 100 mg/mL           | 1 g    |
| Sterile PBS | n/a                  | 10 mL  |

**Note:** Store at 4°C and warm to 20°C–25°C before use.
**Gonadotrophins**

PMSG is prepared as below.

| Reagent   | Final concentration | Amount |
|-----------|---------------------|--------|
| PMSG      | 5 IU/0.1 mL         | 5,000 IU |
| Sterile PBS | n/a                | 100 mL |

*Note:* Store in single-use aliquots of 1 mL at −20°C and warm to 20°C–25°C before use.

hCG is prepared as below.

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| hCG     | 5 IU/0.1 mL         | 10,000 IU |
| Sterile PBS | n/a                | 200 mL |

*Note:* Store in single-use aliquots of 1 mL at −20°C and warm to 20°C–25°C before use.

**Hyaluronidase solution**

| Reagent    | Final concentration | Amount |
|------------|---------------------|--------|
| Hyaluronidase | 0.1%              | 10 mg  |
| M2 medium  | n/a                 | 10 mL  |

*Note:* Store in single-use aliquots of 1 mL at −20°C and warm to 37°C before use.

**Membrane-permeabilized solution (MPs)**

Immunofluorescence (IF) MPs is prepared as below.

| Reagent         | Final concentration | Amount |
|-----------------|---------------------|--------|
| 10% Triton X-100 | 0.5%               | 0.5 mL |
| Sterile PBS     | n/a                 | 9.5 mL |

*Note:* Store at 4°C and warm to 20°C–25°C before use.

**Washing buffer**

IF washing buffer is prepared as below.

| Reagent    | Final concentration | Amount |
|------------|---------------------|--------|
| Tween 20   | 0.1%                | 10 μL  |
| 10% Triton X-100 | 0.01%             | 10 μL  |
| Sterile PBS | n/a                 | 9.98 mL |

Dye staining washing buffer is prepared as below.

| Reagent   | Final concentration | Amount |
|-----------|---------------------|--------|
| BSA       | 0.1%                | 0.01 g |
| Sterile PBS | n/a               | 10 mL  |

*Note:* Store at 4°C and warm to 20°C–25°C before use.
Blocking buffer
IF blocking buffer is prepared as below.

| Reagent           | Final concentration | Amount   |
|-------------------|---------------------|----------|
| BSA               | 1%                  | 0.1 g    |
| Tween 20          | 0.1%                | 10 µL    |
| 10% Triton X-100  | 0.01%               | 10 µL    |
| Sterile PBS       | n/a                 | 9.98 mL  |

*Note:* Store at 4°C and warm to 20°C–25°C before use.

Fixative
Fixative for chromosome spreading is prepared as below.

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| 4% paraformaldehyde      | 1%                  | 250 µL   |
| 10% Triton X-100         | 0.15%               | 15 µL    |
| Sterile PBS              | n/a                 | 735 µL   |

*Note:* Prepare just before use, and adjust pH to 9.2.

Human tubal fluid (HTF) medium
Two drops of 300 µL HTF for sperm capacitation and several drops of 50 µL HTF for fertilization are made in a dish with 35 mm diameter, covered with mineral oil and equilibrated in the incubator at 37°C with atmosphere of 5% CO₂ for 12–16 h before use (Figure 1).

Potassium simplex optimized medium (KSOM) medium
One drop of 50 µL KSOM for in vitro embryo culture is made in a dish with 35 mm diameter, covered with mineral oil and equilibrated in the incubator at 37°C with atmosphere of 5% CO₂ for 12–16 h before use.

Equipment
Turn on the stereo microscope and the heating stage (37°C) 5 min ahead of time. Prepare fine-tipped forceps, mouth pipette, and sterile alcohol cottons (Figure 2).

STEP-BY-STEP METHOD DETAILS
Collection of ovulated oocytes

⊙ Timing: 20 min

Before starting:

- Pre-warm M2 medium to 37°C before use.
- Prepare 1 mg/mL hyaluronidase with M2 medium in advance, and warm it up to 37°C for use.
1. Sacrifice the mice by cervical dislocation after 12–14 h of hCG injection.

△ CRITICAL: The timing to sacrifice the mice after hCG injection is important because it greatly affects the number of oocytes harvested from the oviduct.

2. Hold and cut the oviduct between ovary and uterus out of mice, and place it into M2 medium in the dish on the heating stage.
3. Release ovulated oocytes in M2 medium by tearing a hole in the ampulla of the oviduct with fine-tipped forceps (Figure 3).

**Note:** The ampulla is swollen and translucent such that the oocytes are visible. Be careful not to damage the oocytes by forceps. The heating stage (37°C) should be used to maintain temperature during oocyte manipulation.

4. Remove cumulus cells surrounding oocytes by brief incubation in 1 mg/mL hyaluronidase and then wash oocytes in fresh M2 medium on the heating stage.

⚠️ CRITICAL: Do not exceed 3 min exposure to hyaluronidase as it will damage the oocytes.

Following oocyte collection and fertilization, the effect of NMN supplementation on oocyte and embryo quality can be assessed by 1) Antibody staining and confocal microscopy, go to step 5; 2) Dye staining and confocal microscopy, go to step 12; 3) Chromosome spreading, go to step 15; 4) Sperm binding assay, go to step 21; 5) *In vitro* fertilization and embryo culture, go to step 26.

**Antibody staining and confocal microscopy**

*Timing: 1–1.5 days*

5. Fix 20–30 oocytes in 4% paraformaldehyde (PFA)/PBS (pH 7.4) at 20°C–25°C for 30 min.
Note: It is better to prepare the fixation solution just before use if needed.

6. Permeabilize oocytes in IF MPs at 20°C–25°C for 20 min.

Note: Longer time of permeabilization will damage the oocytes.

7. Block oocytes in IF blocking buffer at 20°C–25°C for 1 h.

Note: This step will reduce the background of the nonspecific binding of the primary antibody.

8. Incubate oocytes with primary antibodies in the blocking buffer at 4°C for 12–16 h.

Alternatives: For some primary antibodies, oocytes can be incubated at 37°C for 4 h. In this case, the background of images might be higher.

Note: The recommended antibody concentration: α-tubulin-FITC (1:200), LCA-FITC (1:100).

9. Wash oocytes in IF washing buffer, and then incubate them with the corresponding secondary antibodies at 20°C–25°C for 1 h.
10. Counterstain oocytes with Hoechst 33342 (10 μg/mL) at 20°C–25°C for 10 min.
11. Mount 10 oocytes in 2 μL of antifade mountant on the microscope slides and seal by coverslip with lanolin. Acquire images under the laser scanning confocal microscope immediately after mounting or store the slide at −20°C.

**Dye staining and confocal microscopy**

© Timing: 1.5–2 h

12. Incubate oocytes in M16 medium containing 500 nM cell permeant MitoTracker Red CMXRos, 2 μM MitoProbe JC-1, 10 μM dichlorofluorescein diacetate (DCFHDA), or 10% Annexin-V-FITC at 37°C with 5% CO2 for 30 min for active mitochondrion staining, mitochondrial membrane potential assessment, DCFH staining, and Annexin-V staining, respectively.

*Note:* The process of dye staining should be completed in dark to avoid the fluorescence quenching.

13. Wash oocytes three times in 0.1% BSA/PBS.
14. Mount 10 oocytes in 2 μL of antifade mountant on the microscope slides and seal by coverslip with lanolin. Acquire images under the laser scanning confocal microscope immediately after mounting or store the slide at −20°C.

△ CRITICAL: For the measurement of fluorescence intensity, acquire images from both control and treated oocytes by following the same staining procedure and setting up the same imaging parameters of the confocal microscope. Apply Image J (NIH, Bethesda, MD, USA) to define a region of interest (ROI) in the images, and determine the average fluorescence intensity per unit area within the ROI. Compare the final average intensities between aged and NMN-supplemented groups.

**Chromosome spreading**

© Timing: 1–1.5 h
15. Warm acidic Tyrode’s solution (pH 2.5) and M2 medium to 37°C.

16. Transfer oocytes into 50 μL Tyrode’s solution for 30 s to remove the zona pellucida (ZP).

**Note:** Too little exposure to the solution will not remove the ZP completely, which will prevent the oocytes from bursting and chromosomes from spreading. Too much exposure will damage and kill the oocytes. Watching the ZP dissolve under the dissection microscope can aid in optimizing the exposure time.

⚠️ **CRITICAL:** Using fresh Tyrode’s solution is critical, as its function declines with age. Use a fresh well of Tyrode’s solution for each group of oocytes to ensure uniform digestion of the ZP. Tyrode’s solution should be aliquoted and stored at –20°C.

17. Wash the oocytes by transferring into warmed M2 medium immediately after ZP removal.

⚠️ **CRITICAL:** Be careful when transferring, as oocytes will easily stick together and to the glass pipette following removal of the ZP. Addition of 0.2% polyvinylpyrrolidone in the medium helps to prevent the oocytes from sticking.

18. Recover oocytes in M2 medium for 10 min at 37°C in a 5% CO2 incubator, and then transfer them with as little M2 medium as possible into one drop of fixative on the microscope slide.

**Note:** The transfer should be done within 30 s, using a microscope without a heating stage.

19. Observe oocyte dissolution under the microscope and leave the slide to air dry at 20°C–25°C for 30 min.

20. After air drying, stain the chromosomes with propidium iodide (PI) for 10 min and acquire images under the laser scanning confocal microscope.

**Sperm binding assay**

⏱ **Timing:** 2–2.5 h
Before starting:

- Equilibrate HTF in the incubator for 12–16 h before use.

21. Release sperm by slicing the cauda epididymides (Figure 4) from 3-month-old male mice (sacrificed by cervical dislocation) with fine-tipped forceps in HTF medium, followed by 1 h of capacitation at 37°C in a 5% CO₂ incubator.

22. Calculate sperm concentration by computer-assisted sperm analysis system, and add $4 \times 10^5$/mL capacitated sperm to 100 μL HTF containing 20–30 ovulated oocytes and 5 two-cell embryos at 37°C with 5% CO₂ for 30 min of binding.

Note: Prepare two-cell embryos as a negative wash control ahead of time, as the sperm cannot bind to embryos.

*Figure 5. Representative images of spindle/chromosome structure in aged and NMN+aged oocytes*
Metaphase I oocytes are immunostained with α-tubulin to show the spindle and counterstained with PI to display the chromosome. Scale bar, 15 μm.

*Figure 6. Representative images of mitochondrial distribution in aged and NMN+aged oocytes*
Metaphase I oocytes are stained with MitoTracker Red to show the mitochondria and counterstained with Hoechst to display the chromosome. Scale bar, 20 μm.
23. Wash sperm binding to the ovulated oocytes with a wide-bore pipette to remove all but 2–6 sperm on the two-cell embryos (negative control).

⚠ CRITICAL: Watch the sperm binding to the oocytes under the dissection microscope when washing them. Too much or too little wash will affect the number of sperm binding to the oocytes.

24. Fix the oocytes with sperm in 4% PFA for 30 min, and stain them with Hoechst 33342 for 10 min.
25. Quantify the bound sperm from z projections acquired by confocal microscope.

**In vitro fertilization and embryo culture**

⊙ Timing: 5 days

Before starting:

- Equilibrate HTF in the incubator for 12–16 h before use.
- Equilibrate KSOM in the incubator for 12–16 h before use.

26. Release sperm by slicing the cauda epididymides from 3-month-old male mice with fine-tipped forceps in HTF medium, followed by 1 h of capacitation at 37°C in a 5% CO₂ incubator.

**Figure 7. Representative images of cortical granule dynamics in aged and NMN+aged oocytes**

Metaphase II oocytes are stained with LCA-FITC to show the cortical granules. Scale bar, 20 μm.
27. Add $4 \times 10^5$/mL capacitated sperm to 100 μL HTF containing 20–30 ovulated oocytes at 37°C with 5% CO₂ for 5 h of incubation (Methods Video S1).

**Note:** The presence of two pronuclei is scored as successful fertilization.

△ **CRITICAL:** Observe the activity of sperm before addition to the oocytes, as it is critical for the success of the fertilization. If the sperm activity is weak, release the sperm from another male mouse immediately. (See Troubleshooting 3)

28. Transfer the fertilized oocytes to a 96-well culture plate containing 100 μL KSOM medium covered with mineral oil at 37°C in a 5% CO₂ atmosphere for in vitro embryo culture for 4 days to the blastocysts.

△ **CRITICAL:** Do not frequently (more than once per day) take embryos out of the incubator to observe the development during the culture, as it will greatly impact the progression of embryonic development. (See Troubleshooting 4)

**EXPECTED OUTCOMES**

The advanced maternal age of female mammals is highly correlated to the decline of oocyte quality, which determines the reproductive performance of females (Balkenende et al., 2020; Deng, 2012; Traub and Santoro, 2010). The strategies to improve the oocyte quality and reproductive performance of females have been a hot research topic in the area of reproductive biology and medicine. Nicotinamide adenine dinucleotide (NAD⁺) is a coenzyme of many enzymes (Gomes et al., 2013; Yoshino et al., 2018; Yoshino et al., 2011), especially the Sirtuin family proteins and it has been implicated in a variety of biological processes such as DNA damage repair, autophagy, adaptive stress responses, genomic stability, and cell survival (Bonkowski and Sinclair, 2016; Croteau et al., 2017; Kennedy et al., 2016). Of note, the decreased NAD⁺ levels with age across many tissues are involved in the aging-related diseases (Bonkowski and Sinclair, 2016; Das et al., 2018; de Picciotto et al., 2016). Therefore, as a precursor metabolic intermediate of NAD⁺, supplementation of NMN is an effective way to recover the NAD⁺ levels and it is likely to be a feasible anti-aging intervention.

Here, we present an efficient protocol to generate high-quality oocytes from the aged mice by *in vivo* supplementation of NMN. In this way, a higher number of ovulated oocytes with reduced...
fragmentation of cytoplasm can be obtained. By fluorescence staining and imaging, key indicators of the oocytes, such as the spindle/chromosome structure, mitochondrial distribution, cortical granule dynamics, and chromosome ploidy, can be assessed. In aged oocytes, a higher frequency of disorganized spindle apparatus with misaligned chromosomes (Figure 5), aggregated clusters of mitochondria in the cytoplasm (Figure 6), discontinuous distribution of cortical granules in the cortex (Figure 7) and more or less number of chromosomes (Figure 8) will be observed. NMN supplementation is expected to restore these defects in aged oocytes (Figures 5, 6, 7, and 8). Additional parameters of oocyte health, specifically reduced level of ROS (Figure 9) and limited occurrence of apoptosis (Figure 10), in NMN-supplemented oocytes can also be evaluated. Moreover, since the decline of oocyte quality is reflected in the impairment of fertilization ability as well (Miao et al., 2018), the sperm binding assay and in vitro fertilization assay can be used to monitor the increased number of sperm binding to the oocytes and elevated fertilization rate in NMN-supplemented oocytes. Lastly, subsequent in vitro embryo culture is expected to further demonstrate the enhanced early embryonic development potential of NMN-supplemented oocytes (Figure 11).

LIMITATIONS
This protocol only describes an in vivo method by NMN injection to improve the oocyte and embryo quality from aged mice. If the researchers are planning to administrate NMN by drinking water or gavage, the optimal dose and time of NMN supplementation should be tested, because the high
The dose of NMN will negatively affect the oocyte and embryo quality. NMN could also be supplemented to the culture medium to promote the oocyte maturation and embryonic development in vitro, the concentration should be optimized.

**TROUBLESHOOTING**

**Problem 1**
Failure to produce positive effects after NMN supplementation

**Potential solution**
The dose of NMN is critical for its impact on the oocyte and embryo quality. Administration of low dose probably has no beneficial effects, but administration of high dose might result in adverse outcomes. Thus, it is necessary to determine the optimal administration dose and time according to the age and strain of the mice. Furthermore, different administration methods, such as intraperitoneal injection, intravenous injection, gavage, and water feeding, might have great divergences in effective doses.

**Problem 2**
Failure to harvest ovulated oocytes from aged mice

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*Figure 10. Representative images of apoptotic oocytes in aged and NMN-aged groups*  
Annexin-V is stained to indicate the apoptotic status of oocytes. Scale bar, 20 μm.
Potential solution
Given that less matured oocytes can be produced in aged mice, as well as the insensitivity of aged mice to the gonadotrophins, higher doses of PMSG and hCG might be needed for maximum superovulation of oocytes. Besides, make sure to collect the oocytes 12–14 h after hCG injection, the inappropriate timing would decrease the number of superovulated oocytes.

Problem 3
Low fertilization rate

Potential solution
The sperm should be released from male mice with the age of at least 10-week-old, otherwise it always leads to the impaired fertilization. The capacitation step is also critical for sperm to acquire the fertilizing ability. Afterwards make sure the sperm swim progressively and that the number of sperm used for fertilization is optimal (2–4 × 10⁵/mL). If dead or motionless sperm are clustered over a half, replace by another male mouse. In addition, the ovulated oocytes should be collected just before use, as the long time incubation in the medium will result in the postovulatory aging and decreased fertilization ability of the oocytes.

Problem 4
Low embryonic development rate

Potential solution
Do not take the embryos out of the incubator frequently to observe the developmental status during in vitro culture, as it will maximize the ambient air exposure to damage the embryos. In addition, the shelf life of the culture medium KSOM is short, make sure to use it prior to expiration.

RESOURCE AVAILABILITY
Lead contact
Requests for further information and resources can be directed to the Lead Contact, Bo Xiong (xiongbo@njau.edu.cn).

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

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100298.

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AUTHOR CONTRIBUTIONS
B.X. conceived, designed, and supervised the study; Y.M., J.C., and Q.G. conducted the experiments; Y.M. and B.X. analyzed the data; Y.M. and B.X. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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