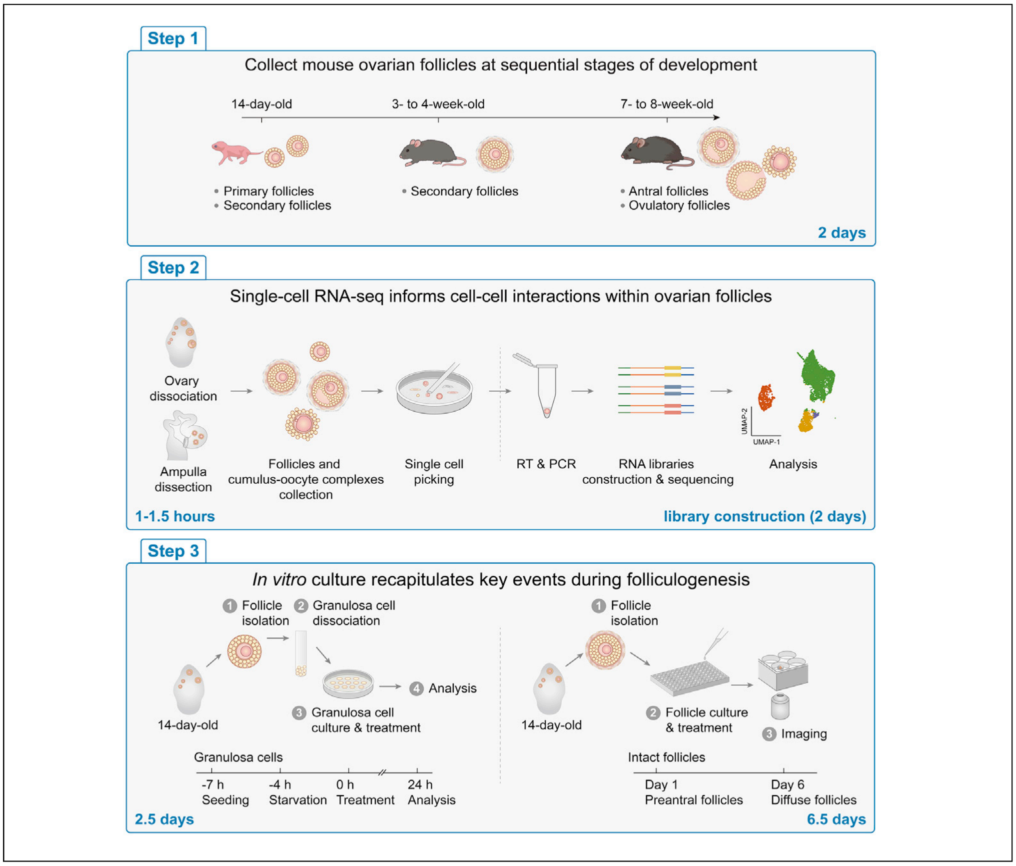
STAR Protocols

CellPress OPEN ACCESS

Protocol

Isolation of mouse ovarian follicles for single-cell RNA-seq and *in vitro* culture



Here, we provide a protocol combing single-cell genomics and functional experiments to study stepwise folliculogenesis. This protocol isolates different developmental stages of mouse ovarian follicles to perform single-cell RNA-seq and *in vitro* culture, allowing to dissect and validate key molecular events in guiding folliculogenesis, thus bringing non-growing oocytes into maturity.

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

Jingjing Qian, Ran Zhu, Rui Yan, Xin Long, Fan Guo

qianjingjing@ioz.ac.cn (J.Q.)
guofan@ioz.ac.cn (F.G.)

Highlights

Detailed protocol for isolating mouse follicles at different stages of development

Manual cell picking to enrich follicular cells rather than ovarian stroma

Relate transcriptome to mechanism by jointing scRNA-seq and *in vitro* culture

Qian et al., STAR Protocols 3, 101537
September 16, 2022
https://doi.org/10.1016/j.xpro.2022.101537

Protocol

Isolation of mouse ovarian follicles for single-cell RNA-seq and *in vitro* culture

Jingjing Qian,[1,2,3,5,*] Ran Zhu,[1,2,3,4] Rui Yan,[1,2,3,4] Xin Long,[1,2,3,4] and Fan Guo[1,2,3,4,6,*]

[1]State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

[2]Institute for Stem Cell and Regeneration, Chinese Academy of Sciences, Beijing 100101, China

[3]Beijing Institute for Stem Cell and Regenerative Medicine, Beijing 100101, China

[4]University of Chinese Academy of Sciences, Beijing 100049, China

[5]Technical contact

[6]Lead contact

*Correspondence: qianjingjing@ioz.ac.cn (J.Q.), guofan@ioz.ac.cn (F.G.)
https://doi.org/10.1016/j.xpro.2022.101537

SUMMARY

Here, we provide a protocol combing single-cell genomics and functional experiments to study stepwise folliculogenesis. This protocol isolates different developmental stages of mouse ovarian follicles to perform single-cell RNA-seq and *in vitro* culture, allowing to dissect and validate key molecular events in guiding folliculogenesis, thus bringing non-growing oocytes into maturity. For complete details on the use and execution of this protocol, please refer to Gu et al. (2019) and Long et al. (2022).

BEFORE YOU BEGIN

Note: Animal work in this protocol was conducted under approval of the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences, China.

Introduction

Ovarian follicles are morphological and functional units of the ovary. In contrast to the broader knowledge of discrete signaling in regulating oogenesis, the holistic signatures of ovarian somatic cells and hierarchical instructions that coordinate folliculogenesis remain to be investigated. Folliculogenesis commences with breakdown of germ cell nests, in which a single oocyte is surrounded by a layer of granulosa cells. As follicles continue to grow, the recruitment of an envelope of theca cells signifies completion of follicle assembly. Genetic and molecular studies have identified fundamental pathways reliant on locally produced molecules in controlling mammalian oogenesis, under the direction of pituitary gonadotropins follicle-stimulating hormone and luteinizing hormone (Edson et al., 2009). The stepwise folliculogenesis is highly instructed. Our recent single-cell studies further provided an insight into epigenetic hierarchy governing development transition of oocytes (Gu et al., 2019; Yan et al., 2021).

It is well known that oocytes are vulnerable to several maternal environmental exposures (Lane et al., 2014), and much progresses have also been made in understanding how the dysfunctional oocyte contributes to offspring disease predisposition (Saben et al., 2016; Risal et al., 2019). However, the mechanisms by which maternal inputs are transmitted to the oocyte remain largely unknown. Within ovarian follicle, reciprocal dialog between somatic cells and oocyte is a prerequisite for oocyte development. In obese mouse models, we recently discovered that deregulated signaling in follicular somatic cells could compromise oocyte quality, explaining impacts of maternal obesity on offspring health (Long et al., 2022).

When dissociating single cells from a whole ovary, stroma predominates. This protocol adopts a hand-pick method to capture oocytes and follicular somatic cells for single-cell RNA-seq for two reasons. Firstly, the number of oocytes is relatively small compared to somatic cells. To focus on how direct crosstalk between theca cells and granulosa cells could interfere manifestation of oocyte phenotype, this protocol starts with isolation and enrichment of mouse ovarian follicles at a succession of developmental stages, followed by manual picking to enrich oocytes and follicular somatic cells. Secondly, diameters of growing and mature oocytes (~30–100 μm) are larger than usual cell type, which is difficult to be captured by 10× chromium, as cell size suggested by 10× chromium is ~30 μm.

This protocol provides an experiment framework and detailed methods combing single-cell RNA-seq and *in vitro* culture to test function of candidate factors identified by single-cell analyses. For further complete information, please refer to our recent work (Gu et al., 2019; Long et al., 2022).

Specification of mice age

Follicles across growing stages predominate in ovaries of mice when puberty begins, and antral follicles can be collected from ovaries of young adulthood (Zheng et al., 2014; Qian and Guo, 2022). Oocytes and ovarian somatic cells contributed by female mice at different ages is a key to capture transcriptome dynamics across folliculogenesis. In this protocol, growing follicles are collected from ovaries of 3- to 4-week-old mice; antral follicles and ovulatory follicles are collected from ovaries of 7- to 8-week-old mice. It is worth noting that although mature oocytes can also be retrieved from mice until 8-month-old (in our experience), in this protocol, 7- to 8-week-old mice are recommended to be used as young adulthood to evade any potential effects arisen by aging.

Note: Experiments using mice must be performed in compliance with all related institutional and governmental rules.

Specification of gonadotropin regimes

In this protocol, oocytes and surrounding somatic cells are retrieved from either ovaries or oviducts. According to the tissue used, different regimes are used for administration of gonadotropins. Ovulatory follicles are retrieved from oviducts to collect metaphase II oocytes and cumulus cells (granulosa cells surrounding the ovulated oocytes), and mice used to collect ovulatory follicles need to be injected with sequential PMSG (pregnant mare serum gonadotropin) and hCG (human chorionic gonadotropin). Growing and antral follicles are dissociated from ovaries to collect growing oocytes and follicular somatic cells, and mice used to collect follicles from ovaries only need to be injected with PMSG.

Dosages of gonadotropin administrated

In this protocol, we prepared PMSG or hCG solution of 50 IU/mL (see in materials and equipment section). According to this, 0.1 mL (5 IU in total) of PMSG and 0.1 mL (5 IU in total) of hCG are used for 3- to 4-week-old mice; 0.2 mL (10 IU in total) of PMSG and 0.2 mL (10 IU in total) of hCG are used for 7- to 8-week-old mice.

Note: Dosages of gonadotropins are based on bodyweight of the mice, and whether inter-strain variability in response to gonadotropins exists should to be considered, in addition to the possible difference in bodyweight among stocks. We used C57BL/6J females in this protocol. The average body weight for C57BL/6J females is 10–15 g at the age of 3- to 4-week, accordingly, 5 IU of gonadotropins are injected to mice at this age; the average body weight is 20–25 g at the age of 7- to 8-week, accordingly, 10 IU of gonadotropins are injected to mice at this age. Whether and to what extent the efficacy of gonadotropins would be compromised in mice older than 6-month remains undetermined. Possibly dosages need to be increased for aged mice, however, care should be taken to avoid potential overdose. Furthermore, in our hands, this correlation between dosages of gonadotropin and body-weight of mice also works well in CD-1(ICR) mice.

Administration of gonadotropins

Timing: ~42–48 h

1. Hold the mouse, pierce through skin and muscle layer at the caudal left of the abdomen to inject PMSG into intraperitoneal cavity by using a disposable 1 mL-sterile syringe equipped with a 27-gauge needle.
2. To collect growing and antral follicles, ovaries are dissected directly 42–44 h after injection of PMSG.
3. To collect ovulatory follicles, 46–48 h after injection of PMSG, hCG is further injected, also through intraperitoneal administration. Oviducts are dissected 15–16 h after injection of hCG.

⚠ CRITICAL: Make sure the entry point of the needle is at the right angle/ below skin and muscle layers. Appearance of a bleb at injection site indicates that the hormone solution was administrated subcutaneously rather than intraperitoneally, and this would compromise oocyte yield.

⚠ CRITICAL: Avoid bladder during intraperitoneal administrations.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Cnmd, Rabbit Polyclonal (1:100)	Thermo Fisher Scientific	Cat# PA5-76974
Anti-Rabbit-Alexa 555, Goat Polyclonal (1:1000)	Thermo Fisher Scientific	Cat# A-21429
Experimental models: Organisms/strains		
Mice: C57BL/6J (wild-type, female, 14-day-old)	Charles River	N/A
Mice: C57BL/6J (wild-type, female, 3- to 4-week-old)	Charles River	N/A
Mice: C57BL/6J (wild-type, female, 7- to 8-week-old)	Charles River	N/A
Chemicals, peptides, and recombinant proteins		
PMSG	ProSpec	Cat# HOR-272
hCG	ProSpec	Cat# HOR-250
Sodium chloride (NaCl)	Sigma-Aldrich	Cat# S9888
Triton X-100	Sigma-Aldrich	Cat# T9284
M2 Medium	Sigma-Aldrich	Cat# M7167
Type IV-S Hyaluronidase	Sigma-Aldrich	Cat# H4272
Tyrode's Solution, Acidic	Sigma-Aldrich	Cat# T1788
Albumin, Acetylated from bovine serum	Sigma-Aldrich	Cat# B2518
ITS Liquid Media Supplement (100 ×)	Sigma-Aldrich	Cat# I3146
EmbryoMax® Nucleosides (100 ×)	Sigma-Aldrich	Cat# ES-008-D
Betaine	Sigma-Aldrich	Cat# 61962-50G
DPBS, no calcium, no magnesium	Gibco	Cat# 14190-144
Leibovitz's L-15 Medium	Gibco	Cat# 11415-064
α-MEM Medium	Gibco	Cat# 12561056
Penicillin-Streptomycin (100 ×)	Gibco	Cat# 15140122
Collagenase, Type IV	Gibco	Cat# 17104019

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
TrypLE Express Enzyme (1 ×)	Gibco	Cat# 12604-021
ProLong Gold Antifade Mountant with DAPI	Thermo Fisher Scientific	Cat# P36935
Nuclease-Free Water	Thermo Fisher Scientific	Cat# AM9932
Dynabeads “MyOne” Streptavidin C1	Thermo Fisher Scientific	Cat# 65002
UltraPure 1 M Tris-HCl, pH 7.5	Thermo Fisher Scientific	Cat# 15567-027
Superscript II reverse transcriptase	Thermo Fisher Scientific	Cat# 18064071
dNTP mix	Thermo Fisher Scientific	Cat# R0192
Recombinant RNase Inhibitor (40 U/mL)	Takara Bio	Cat# 2313B
Ampure XP Beads	Beckman Coulter	Cat# A63882
Magnesium chloride (MgCl2)	VMR	Cat# J364-100G
2 × KAPA HiFi HS ReadyMix	Roche	Cat# 7958935001
Fetal Bovine Serum, New Zealand	VISTECH	Cat# SE200-ES
Tissue-Tek® O.C.T. Compound	Sakura	Cat# 4583
Adhesion Microscope Slides	CITOTEST	Cat# 80312-3161
recombinant human FSH (300 IU/0.5 mL)	Merck Serono	Cat# #Gonal-f
Ethanol, absolute	In house	N/A
Critical commercial assays		
DNA Clean & Concentrator-5 (Capped)	Zymo Research	Cat# D4014
Qubit dsDNA High-Sensitivity Kit	Invitrogen	Cat# Q32851
NEBNext Ultra II DNA Library Prep Kit	New England Biolabs	Cat# E7645L
NEBNext Multiplex Oligos for Illumina	New England Biolabs	Cat# E7335S
Oligonucleotides		
TSO primer: AAGCAGTGGTATCAAC GCAGAGTACATrGrG+G	Integrated DNA Technologies	N/A
Oligo-dT primers: TCAGACGTGTGCTCTT CCGATCTAACGTGATNNNNNNNNXXXXXXXX TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	Integrated DNA Technologies	N/A
ISPCR primer: AAGCAGTGGTATCAACGCAGAGT	Integrated DNA Technologies	N/A
3′P2 primer: GTGACTGGAGTTC AGACGTGTGCTCTTCCGATC	Integrated DNA Technologies	N/A
QP2 primer: CAAGCAGAAGACGGCATACGA	Integrated DNA Technologies	N/A
Short Universal primer: AATGATACGGCGACCAC CGAGATCTACACTCTTTCCCTACACGAC	Integrated DNA Technologies	N/A
Other		
Abrasive stone	In house	N/A
Centrifuge, refrigerated	Eppendorf	5425R
Confocal Microscope	Carl Zeiss	LSM880
Micro Scissors	JinZhong, Shanghai	Cat# W10110
Micro Forceps	JinZhong, Shanghai	Cat# WA3090
Fine Forceps	JinZhong, Shanghai	Cat# J3C040
Needles, 27-gauge	BD Microlance	Cat# BD300635
Rubber tubing	Sigma-Aldrich	Cat# A5177
QSP 200, Filtered Tips	Thermo Fisher Scientific	TF140-200-Q
ThermoMixer F1.5	Thermo Fisher Scientific	EP5384000020
DNA LoBind Tubes, 1.5 mL	Eppendorf	Cat# 0030108051
DNA LoBind Tubes, 2.0 mL	Eppendorf	Cat# 0030108078
Qubit Assay Tubes, 0.5 mL	Axygen	Cat# PCR-05-C
0.2-mL Thin Wall PCR Tubes with Flat Cap	Axygen	Cat# PCR-02-C
15-mL High Clarity PP Centrifuge Tube	Corning	Cat# 352096
50-mL High Clarity PP Centrifuge Tube	Corning	Cat# 352070
Focused-ultrasonicator	Covaris	Cat# M220
Fragment analyzer	AATI	N/A
microTUBE Snap-Cap	Covaris	Cat# 520045

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Vortex mixer	SCILOGEX	Cat# SCI-VS
Magnetic Rack for 0.2-mL PCR tubes	Thermo Fisher Scientific	Cat# 492025
Qubit Fluorometer	Thermo Fisher Scientific	Cat# Q33216
Thermal Cycler	Thermo Fisher Scientific	ProFlex
CO_2 incubator	Esco	CCL-170B
Conical Centrifuge Tubes	BD Falcon	Cat# 352097
Petri Dishes, 35 mm × 10 mm	BD Falcon	Cat# 351008
Pre-Separation Filter, 30 μm	Miltenyi Biotec	130-041-407
96-well Cell Culture Cluster, Round Bottom	Corning	Cat# 3879
Dissecting microscope	Nikon	SMZ1270

MATERIALS AND EQUIPMENT

Preparation of gonadotropins

Timing: ~1 h

PMSG (pregnant mare serum gonadotropin) work solution

Reagent	Final concentration	Amount
PMSG	50 IU/mL	1,000 IU
0.9% sodium chloride	1 ×	20 mL

Store at −20°C for 6 months.

Gonadotropins are suppled as lyophilized powder. Reconstitute 1 vial (1,000 IU) of PMSG with 20 mL 0.9% sodium chloride to obtain a work solution of 50 IU/mL, filter through a 0.2 μm filter to sterilize, aliquot, and store at −20°C. Thaw PMSG work solution at room temperature 0.5 h before use. Avoid refreezing.

hCG (human chorionic gonadotropin) work solution

Reagent	Final concentration	Amount
hCG	50 IU/mL	12,500 IU
0.9% sodium chloride	1 ×	250 mL

Store at −20°C for 6 months.

Reconstitute 1 vial (12,500 IU) of hCG with 250 mL 0.9% sodium chloride to obtain a work solution of 50 IU/mL, filter through a 0.2 μm filter to sterilize, aliquot, and store at −20°C. Thaw hCG work solution at room temperature 0.5 h before use. Avoid refreezing.

Preparation of digestive enzymes

Timing: ~1 h

Hyaluronidase stock solution

Reagent	Final concentration	Amount
Hyaluronidase (lyophilized powder)	10 mg/mL	30 mg
M2 medium	1 ×	3 mL
Total	N/A	3 mL

Store at −20°C for 6 months.

Reconstitute 1 vial (30 mg) of hyaluronidase with 3 mL M2 medium to first obtain a stock solution at 10 mg/mL, filter through a 0.2 μm filter to sterilize, aliquot. Avoid refreezing.

Hyaluronidase work solution		
Reagent	Final concentration	Amount
Hyaluronidase (10 mg/mL)	0.3 mg/mL	0.3 mL
M2 medium	1 ×	9.7 mL
Total	N/A	**10 mL**

Store at 4°C for 1 week.
Dilute to 0.3 mg/mL with M2 medium as work solution. Prewarm at 37°C for 5 min right before use.

Collagenase stock solution		
Reagent	Final concentration	Amount
Collagenase, Type IV (lyophilized powder)	10 mg/mL	100 mg
M2 medium	1 ×	10 mL
Total	N/A	**10 mL**

Store at −20°C for 6 months.
Reconstitute 100 mg Type IV Collagenase with 10 mL M2 medium to first obtain a stock solution at 10 mg/mL, filter through a 0.2 μm filter to sterilize, aliquot. Avoid refreezing.

Collagenase work solution		
Reagent	Final concentration	Amount
Collagenase, Type IV (10 mg/mL)	1 mg/mL	1 mL
M2 medium	1 ×	9 mL
Total	N/A	**10 mL**

Store at 4°C for 2 weeks.
Dilute to 1 mg/mL with M2 medium as work solution. Prewarm at 37°C for 5 min right before use.

Single-cell RNA-seq reagents setup

Lysis buffer		
Reagent	Final concentration	Amount
RNase Inhibitor (40 U/μL)	1 U/μL	0.05 μL
1% Triton X-100	0.475%	0.95 μL
Barcode oligo-dT primer (10 μM)	0.3 μM	0.15 μL
Nuclease-free water	N/A	0.35 μL
dNTP (10 mM)	1 mM each	0.5 μL
Total	N/A	**2.0 μL**

Prepare right before use.

Elution buffer		
Reagent	Final concentration	Amount
Tris-HCl (1 M, pH 7.5)	10 mM	0.5 mL
Nuclease-free water	N/A	49.5 mL
Total	N/A	**50 mL**

Store at 4°C for 6 months.

Biotinylated index primers	
Primer	Sequence
Index-primer-#1	/Biotin/CAAGCAGAAGACGGCATACGAGA TCTCTACGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC

(*Continued on next page*)

Continued	
Primer	Sequence
Index-primer-#2	/Biotin/CAAGCAGAAGACGGCATACGAG ATGCTACCGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
Index-primer-#3	/Biotin/CAAGCAGAAGACGGCATACGAG ATGCTCATGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC
Index-primer-#4	/Biotin/CAAGCAGAAGACGGCATACGAG ATTGCCATGTGACTGGAGTT CAGACGTGTGCTCTTCCGATC

RT mix		
Reagent	Final concentration	Amount
SuperScript II reverse transcriptase (200 U/μL)	10 U/μL	0.25 μL
RNase inhibitor (40 U/μL)	1 U/μL	0.125 μL
Superscript II first-strand buffer (5 ×)	1 ×	1.0 μL
DTT (0.1 M)	5 mM	0.25 μL
Betaine (5 M)	1 M	1.0 μL
$MgCl_2$ (1 M)	6 mM	0.03 μL
TSO (100 μM)	1 μM	0.05 μL
Nuclease-free water	N/A	0.145 μL
Total	**N/A**	**2.85 μL**
Prepare right before use.		

Index PCR mix		
Reagent	Final concentration	Amount
KAPA HiFi HotStart ReadyMix (2 ×)	1 ×	25 μL
ISPCR primers (10 μM)	0.4 μM	2 μL
Biotin index primer (10 μM)	0.4 μM	2 μL
Nuclease-free water	N/A	0.25 μL
Total	**N/A**	**29 μL**
Prepare right before use.		

Preamplification mix		
Reagent	Final concentration	Amount
KAPA HiFi HotStart ReadyMix (2 ×)	1 ×	6.25 μL
ISPCR primers (10 μM)	0.2 μM	0.25 μL
3′P2 (10 μM)	0.6 μM	0.75 μL
Nuclease-free water	N/A	0.25 μL
Total	**N/A**	**7.5 μL**
Prepare right before use.		

Culture medium setup

Follicle culture medium		
Reagent	Final concentration	Amount
α-MEM	1 ×	45.992 mL
Fetal Bovine Serum	5%	2.5 mL
Penicillin-Streptomycin (100 ×)	1 ×	0.5 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
ITS Liquid Media Supplement (100 ×)	1 ×	0.5 mL
EmbryoMax® Nucleosides (100 ×)	1 ×	0.5 mL
recombinant human FSH (300 IU/0.5 mL)	100 mIU/mL	0.008 mL
Total	N/A	50 mL
Store at 4°C for up to 2 weeks.		

Handling medium		
Reagent	Final concentration	Amount
L15 medium	1 ×	44.5 mL
Fetal Bovine Serum	10%	5 mL
Penicillin-Streptomycin (100 ×)	1 ×	0.5 mL
Total	N/A	50 mL
Store at 4°C for up to 2 weeks.		

Granulosa cells growth medium		
Reagent	Final concentration	Amount
α-MEM	1 ×	46 mL
Fetal Bovine Serum	5%	2.5 mL
Penicillin-Streptomycin (100 ×)	1 ×	0.5 mL
ITS Liquid Media Supplement (100 ×)	1 ×	0.5 mL
EmbryoMax® Nucleosides (100 ×)	1 ×	0.5 mL
Total	N/A	50 mL
Store at 4°C for up to 2 weeks.		

Granulosa cells starvation medium		
Reagent	Final concentration	Amount
α-MEM	1 ×	48.5 mL
Penicillin-Streptomycin (100 ×)	1 ×	0.5 mL
ITS Liquid Media Supplement (100 ×)	1 ×	0.5 mL
EmbryoMax® Nucleosides (100 ×)	1 ×	0.5 mL
Total	N/A	50 mL
Store at 4°C for up to 2 weeks.		

STEP-BY-STEP METHOD DETAILS

This protocol provides a framework to decipher principles of folliculogenesis. To reach this goal, single-cell genomics followed by well-controlled *in vitro* cultures to relate comprehensive transcriptome data to causal mechanisms. This protocol involves 5 sections. Section 1: preparation of single metaphase II oocytes and cumulus cells from ovulatory follicles and section 2: preparation of single growing oocytes, fully grown oocytes and ovarian somatic cells from growing and antral follicles are detailed methods to prepare follicular cell suspension for single-cell RNA-seq in section 3: single-cell RNA-seq library construction. Section 4: isolating preantral follicles and culturing up to maturation and section 5: isolating immature granulosa cells for in vitro culture offer detailed methods for follicle and follicular cell culture and depend on preference of researchers to follow this protocol.

Section 1: Preparation of single metaphase II oocytes and cumulus cells from ovulatory follicles.

Section 2: Preparation of single growing oocytes, fully grown oocytes and ovarian somatic cells from growing and antral follicles.

Section 3: Single-cell RNA-seq library construction.

Section 4: Isolating preantral follicles and culturing up to maturation.

Section 5: Isolating immature granulosa cells for *in vitro* culture.

⚠ CRITICAL: When preparing a hand-pulled micro capillary pipette for manipulating oocytes, the internal diameter of the opening should be slightly larger than an oocyte (~100–120 μm); and the opening tip need to be polished by swiftly touching a flame. This is important for removal of zona by brief Tyrode's exposure without damaging oocytes. Handling these samples needs much practice.

Section 1: Preparation of single metaphase II oocytes and cumulus cells from ovulatory follicles

Timing: ~0.5–1 h

1. Approximately 15–16 h after hCG injection (Figure 1A), humanely cull the mouse and place on an absorbent pad. Open abdominal cavity, cut peritoneum, and put back digestive system to expose reproductive organs, including two ovaries, two oviducts and two horns of uterus for each mouse.
2. Grasp the upper end of uterus and gently pull away from body, make the first cut between uterus and utero-tubal junction (a narrow tube connects uterus and oviduct). Relocate the forceps near to utero-tubal junction, then a second cut is made between infundibulum of oviduct (the opening of oviduct) and ovary (Figure 1B). Repeat to cut off the other side of oviduct. Put oviducts into a drop of M2 medium in a 35-mm Petri dish.

Note: Prewarm M2 medium at 37°C for 5 min right before use.

⚠ CRITICAL: Pay attention not to break oviduct during dissection.

3. Use a pair of fine forceps to grasp isthmus and hold oviduct on the bottom of the Petri dish, immerse the oviduct in a drop of M2 medium. Use the second pair of fine forceps to tear ampulla (Figure 1C1) where cumulus-oocyte complexes are located, releasing the clutches of cumulus-oocyte complexes (Figure 1C2).

⚠ CRITICAL: In response to injection of gonadotropins, ampulla is usually swollen and transparent to be easily identified under a dissecting microscope.

4. Use a 20-μL tip to dispense 2 drops (20-μL) of hyaluronidase medium and 2 drops (20-μL) of M2 medium on the bottom of a 35-mm Petri dish (Figure 1D).
5. Transfer the collected cumulus-oocyte complexes by using forceps into the first drop of hyaluronidase. Under a dissecting microscope, incubate cumulus-oocyte complexes in hyaluronidase solution for 2–3 min (no more than 5 min) until the cumulus cells start to loosen. Oocytes surrounded by more sticky cumulus cells should be transferred by a finely pulled glass pipette into a second fresh drop of hyaluronidase, observe until cumulus cells fall off.

Note: For oocyte surrounded by cumulus cells that are easily shed, only the first drop of hyaluronidase is needed. For stickier cumulus cells, transfer of cumulus-oocyte complexes into the

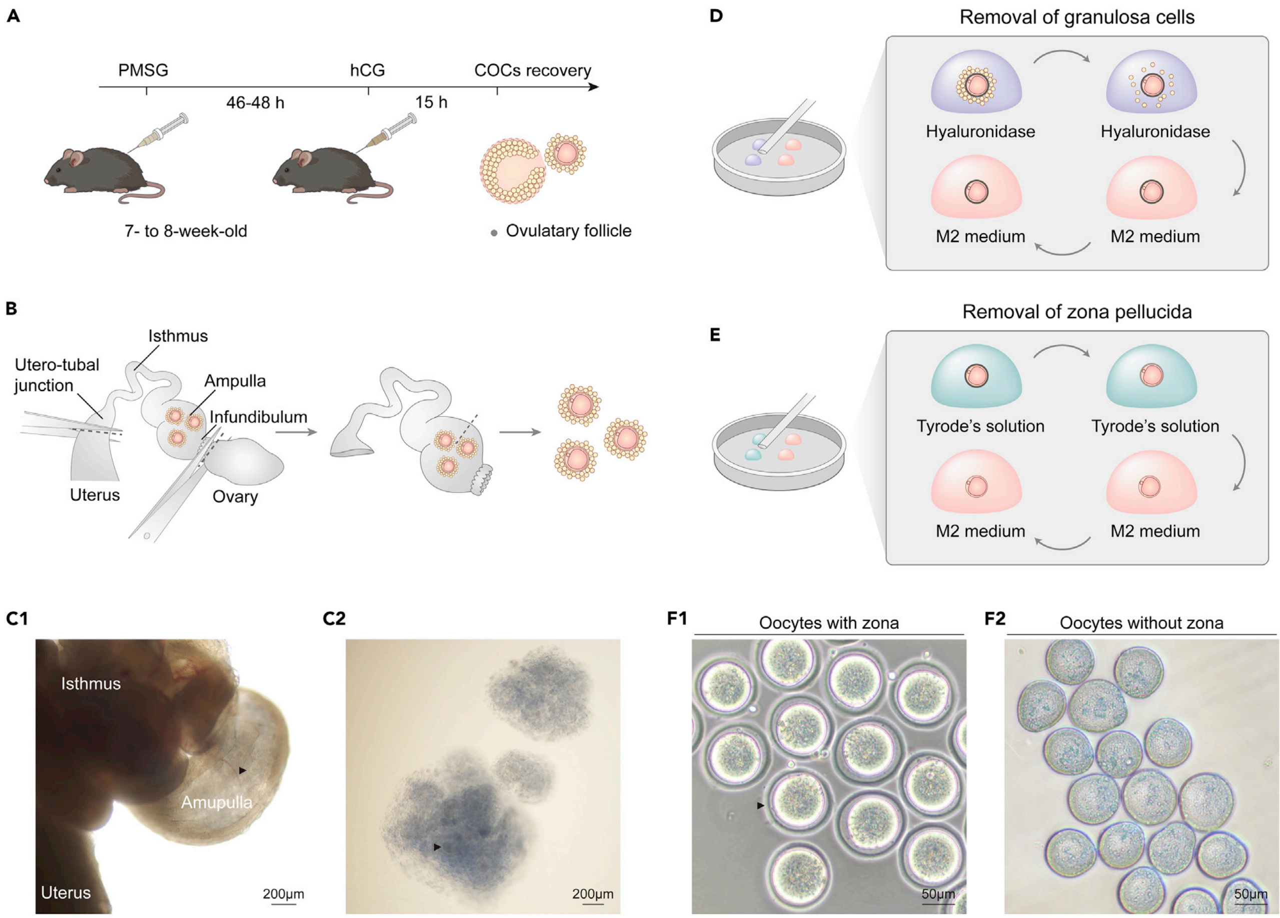


Figure 1. Preparing single-cell suspensions from ovulatory follicles

(A) Age of mice, time scheme of PMSG and hCG injections, COCs recovery. COCs, cumulus-oocyte complexes.
(B) Dissection of ampulla to recovery COCs, dashed lines indicate the positions to make cuts.
(C1) A representative picture showing the swollen ampulla, black arrowhead indicates an oocyte.
(C2) A representative picture showing cumulus-oocyte complexes retrieved from one female, black arrowhead indicates an oocyte, Scale bar, 200 μm.
(D) Sequential steps to dissociate granulosa cells.
(E) Sequential steps to remove zona pellucida.
(F1) A representative picture showing oocytes with zona before Tyrode's treatment, black arrowhead indicates zona.
(F2) A representative picture showing oocytes without zona after Tyrode's treatment. Scale bar, 50 μm.

second fresh drop of hyaluronidase is important to minimize exposure time in hyaluronidase. Pipette up and down with Pasteur pipette for a few times helps.

6. Use a hand pipette, pick up the dissociated individual oocytes and granulosa cells into a fresh drop of M2 medium and perform two washes in M2 medium droplets.
7. Use a 20-μL tip to dispense 2 drops (20-μL) of Tyrode's medium and 2 drops (20-μL) of M2 medium on the bottom of a 35-mm Petri dish (Figure 1E).
8. Transfer a group of 10–12 oocytes into the first Tyrode's drop, pipette up and down for 3–5 times, further transfer the oocytes into the second Tyrode's drop, observe until zona pellucida gradually becomes thinning and disappears. Representative pictures of oocytes with zone before Tyrode's treatment and without zona after Tyrode's treatment are shown in Figures 1F1 and F2, respectively.

Note: Expunge the remaining medium in the hand Pasteur pipette into a blank Petri dish (for waste collection) before refill it with new Tyrode's medium, as any trance of M2 carried would neutralize Tyrode's effects.

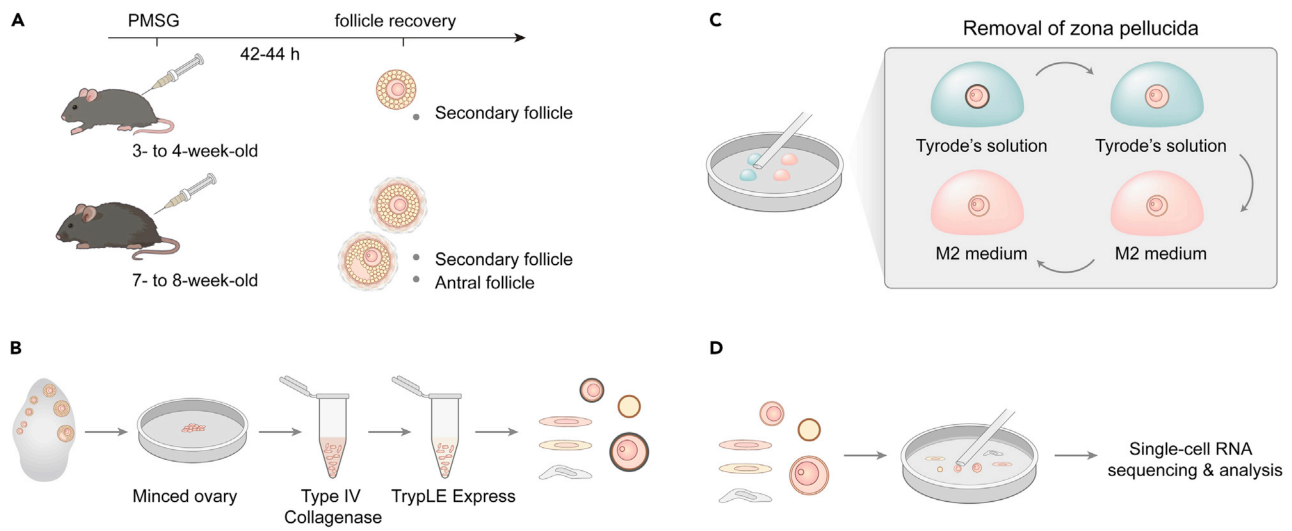


Figure 2. Preparing single-cell suspensions from growing and antral follicles
(A) Age of mice, time scheme of PMSG injection, secondary and antral follicle recovery.
(B) Dissection of ovary to recovery ovarian cells.
(C) Sequential steps to remove zona pellucida.
(D) Individual oocytes and ovarian somatic cells are picked for single-cell RNA-seq.

9. Use a Pasteur pipette to pick up the zona-free oocytes into a drop of M2 medium to wash, then repeat once in another drop of M2.
10. Use a 20-µL tip to dispense several drops (20-µL) of 0.1% bovine serum albumin (BSA) (dissolved in 1 × DPBS).
11. Use a Pasteur pipette to transfer granulosa cells (from step 6) and zona-free oocytes (from step 9) sequentially through two drops of 0.1% BSA (dissolved in 1 × DPBS) to perform washes.
12. Pick single oocytes or granulosa cells into lysis buffer that is ready to prepare single-cell cDNA (complementary DNA).

Note: Promptly pick disaggregated single cells into lysis buffer and keep them immediately on ice.

Section 2: Preparation of single growing oocytes, fully grown oocytes, and ovarian somatic cells from growing and antral follicles

Timing: ~1–1.5 h

13. Approximately 42–44 h after injection of a single shot of PMSG, collect secondary follicles from ovaries of 3- to 4-week-old mice; collect secondary and antral follicles from ovaries of 7- to 8-week-old mice (Figure 2A).
14. Ovaries are dissected, carefully trimmed, and chopped into 0.5-mm^3 cubes.

Note: The following digestive volume is recommended for 2 ovaries to ensure optimal performance.

15. Use a two-stage of digestion procedure to prepare single cell suspension. Digest the minced ovaries in 400µL 1 mg/mL Type IV collagenase at 37°C under 1,000 × rpm for 20 min in a ThermoMixer (Figure 2B).
16. Spin at 300 × g for 3 min at room temperature and discard the resulting supernatant.

17. Resuspend the pellet in 400 μL of prewarmed TrypLE Express at 37°C under 1,000× rpm for 10–15 min (Figure 2B). Observe under a dissecting microscope to monitor status of cells during dissociation. It is important to gently homogenize by intermitting pipetting every 7–8 min during digestion.

⚠ CRITICAL: Visualize cell suspensions is important for evaluation of cell viability. If cell aggregates are observed, one should perform further gentle pipetting; if debris is observed, one should perform filtering. Once single-cell suspensions are obtained, promptly process to stop digestion. Avoid over incubation in digestive enzymes to avoid damaging cells or lysing.

18. Stop digestion by adding 400μL of prewarmed M2 medium, pipette the resulting cell suspension and transfer it into a 35-mm Petri dish, at this point single somatic cells or single oocytes could be obtained.
19. Use a 20-μL tip to dispense 2 drops (20-μL) of M2 medium on the bottom of a 35-mm Petri dish.
20. Use a hand glass pipette, pick up the dissociated individual oocytes and somatic cells into a fresh drop of M2 medium and perform two washes in M2 medium droplets.
21. Use a 20-μL tip to dispense 2 drops (20-μL) of Tyrode's medium and 2 drops (20-μL) of M2 medium on the bottom of a 35-mm Petri dish (Figure 2C).
22. Remove zona pellucida encapsulating growing oocytes by Tyrode's solution, and then wash zona-free oocytes immediately through 2 drops of M2 medium.
23. Wash somatic cells (from step 20) and zona-free oocytes (from step 22) in 0.1% BSA (dissolved in 1 × DPBS).
24. Pick individual zona-free oocytes or somatic cells into lysis buffer that is ready to prepare single-cell cDNA (Figure 2D).

Note: Promptly pick disaggregated single cells into lysis buffer and keep them immediately on ice.

Section 3: Single-cell RNA-seq library construction

⏲ Timing: ~2 days

25. Individual single cells from step 12 or step 24 are manually picked into individual 0.2-mL PCR tubes by a hand glass pipette, vortex thoroughly for 30 s, incubate at 72°C for 3 min in a Thermal Cycler, then immediately place on ice.
26. Add 2.85 μL reverse transcription (RT) mix to each sample.
27. Vortex briefly and spin down at 1,000 × g for 1 min at 4°C, perform PCR in a Thermal Cycler as below:

RT PCR cycling conditions

Steps	Temperature	Time	Cycles
1	25°C	5 min	1
2	42°C	60 min	1
3	50°C	30 min	1
4	70°C	10 min	1
5	4°C	Hold	N/A

28. Add 7.5 μL PCR preamplification mix to each reaction.
29. Vortex briefly and spin down at 1,000 × g for 1 min at 4°C, perform PCR in a Thermal Cycler as below to amplify cDNA:

Preamplification PCR cycling conditions			
Steps	Temperature	Time	Cycles
1	95°C	3 min	1
2	98°C	20 s	4
3	65°C	30 s	
4	72°C	5 min	
5	98°C	20 s	16
6	67°C	15 s	
7	72°C	5 min	
8	72°C	5 min	1
9	4°C	Hold	N/A

30. Pool 4 µL of each cDNA sample with different barcodes (up to 96 barcodes) together.
31. Purify once with Zymo DNA Clean and Concentrator-5 Kit according to manufacturer's protocol, elute in 50 µL elution buffer. Purify twice with 0.8 × Ampure XP beads, elute in 21 µL elution buffer.
32. Check size distribution of cDNA samples by a Fragment Analyzer (AATI). The profile of a successful cDNA sample should have a peak at around 1.5 kb (see expected outcomes).
33. Use 30–40 ng cDNAs as template, add 29 µL index PCR mix, with Biotin index primer to perform a second amplification.
34. Perform PCR in a Thermal Cycler as below:

Index PCR cycling conditions			
Steps	Temperature	Time	Cycles
1	95°C	3 min	1
2	98°C	20 s	3
3	67°C	15 s	
4	72°C	5 min	
5	72°C	5 min	1
6	4°C	Hold	N/A

35. The biotinylated cDNAs are purified once with 0.8× Ampure XP beads, elute in 30 µL elution buffer.

Pause point: The cDNA samples can be stored at −20°C or −80°C for 8 weeks or longer.

36. Shear cDNAs into 300-bp fragments by an ultrasonicator (Covaris) according to manufacturer's protocol.
37. Purify once with Zymo DNA Clean and Concentrator-5 Kit according to manufacturer's protocol, elute in 50 µL elution buffer. Enriched by using Dynabeads® MyOne™ Streptavidin C1.
38. Construct RNA-seq libraries by using NEBNext UltraII DNA Library Prep Kit for Illumina according to manufacturer's instructions. Use QP2 primer and Short Universal Primer to conduct final library PCR amplification.
39. Pool the final RNA-seq libraries together, measure concentration by the Qubit Fluorometer, check size distribution and process to sequence. The expected average size of final libraries is around 200–800 bp, with a peak occurs at around 400 bp. The yield of the library is usually more than 100 ng.

Pause point: The RNA libraries can be stored at −20°C or −80°C for 8 weeks or longer.

For space limitation, we do not provide data analysis details for scRNA-seq data, which covers many aspects by a well-designed project. For detailed computational methods, tools and a comprehensive guide for analysis of scRNA sequencing data, please refer to (Andrews et al., 2021).

Section 4: Isolating preantral follicles and culturing up to maturation

Timing: ~6.5 days

Note: How different systems should be chosen according to aim of the study is reviewed in (Simon et al., 2020). In this protocol, to trace growth of each individual follicle, the selected follicles are cultured singly into each well of a 96-well microplate; to avoid any perturbations to lyophilic steroids signalling, the follicles are cultured without mineral oil overlay.

To verify candidate factors instructing granulosa-theca interactions or stepwise transition across folliculogenesis, prepuberty mice at 14 days are used to isolate immature follicles for culture. At this age, early preantral follicles predominate, only a few follicles reach antral stage, no corpus lutea forms yet.

40. Prepare culture medium for follicle culture (see in materials and equipment section).
41. Sterilize surgery tools and pipettes by heating them in a sterilization oven in advance.
42. Dissect ovaries from 14-day-old female mice, trim and transfer them into L15 medium supplied with 10% FBS and 1 × penicillin-streptomycin.

Note: Make sure the following steps to dissociate follicles for culture are performed in sterile conditions.

43. Transfer ovaries to a laminated-flow hood, immerse them in L15 medium supplied with 10% FBS and 1 × penicillin-streptomycin (15–20 μL medium for 2 ovaries), and mechanically chop into small pieces with a metal blade.

Note: To preserve follicular integrity and investigate intra-follicle cell-cell interactions, preantral follicles are released mainly by mechanical dissection with only brief collagenase treatment, rather than enzymic digestion.

44. Incubate minced ovary tissue in 1 mg/mL Type IV collagenase, at 37°C under 1000× rpm for 10–15 min in a ThermoMixer, to dissociate connective tissues (Figure 3A). A representative picture showing chopped ovary tissue transferred into Type IV collagenase at the start of digestion (Figure 3B).
45. Spin at 300 × g for 3 min at room temperature, resuspend the resulting pellet in L15 medium and transfer them into a 35-mm Petri dish.
46. Released individual intact follicles are manually picked by using a hand pipette, based on two criteria: (1) with diameters around 120 μm; (2) oocyte located centrally within the follicle and is surrounded by 1–2 layers of granulosa cells, a basal membrane, and a layer of thecal cells (Figure 3C).
47. Under a dissecting microscope, pool the picked follicles and sequentially wash them through three 50-μL drops of L15 medium by a hand pipette, then randomly assign to different treatment groups.

Note: Perform washes <30 min if possible.

48. Use a 20-μL tip to dispense a 20-μL drop of follicle culture medium in each well of a 96-well plate. Culture the follicles singly in each well of a 96-well plate in an incubator at 37°C containing 5% CO_2, designating as day 1 of culture.
49. On day 2 of culture, further add 10 μL medium to each follicle droplet; thereafter refresh 10 μL of old medium with fresh medium in each follicle droplet every other day.

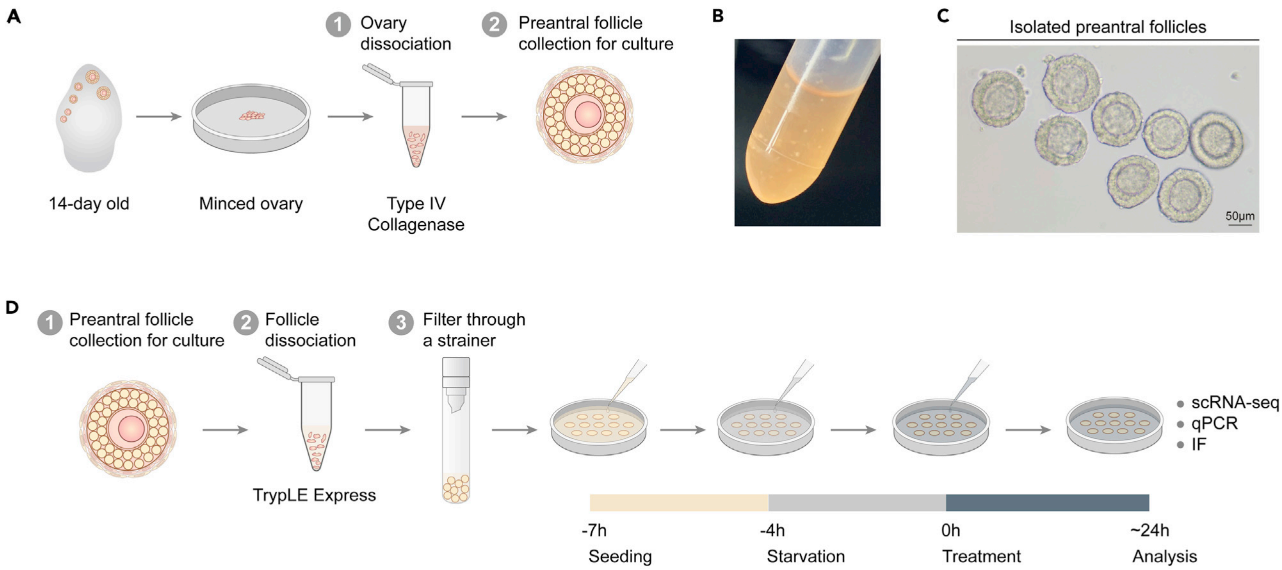


Figure 3. Isolating immature follicles and granulosa cells for *in vitro* culture

(A) Age of mice, dissection of ovary to recovery preantral follicles.

(B) A representative picture showing chopped tissue transferred into Type IV collagenase at the start of digestion.

(C) A representative picture showing the isolated preantral follicles, Scale bar, 50 μm.

(D) Dissociation of immature follicles to isolate granulosa cells for *in vitro* culture. IF, immunofluorescence.

50. Perform morphological evaluation under a microscope every day until day 6 of culture.

Note: In normal conditions, follicles can growth and expand to form a well-organized structure onset of antrum-like cavity formation. In this *in vitro* setting, phenotypes like initiation of granulosa-cell death under certain treatment could suggest a role of the treatment. We have previously used this system to study function of Notch signalling pathway in affecting folliculogenesis (Gu et al., 2019).

Alternatives: Morphological evaluation provides a convenient, time-sequential while relatively gross assessment of follicle growth. If needed, histological characteristics of sections of the cultured follicles could provide a detailed phenotype, for example, whether and when atresia and cell death of granulosa cells initiates (Cortvrindt et al., 1996).

Section 5: Isolating immature granulosa cells for *in vitro* culture

Timing: ~2.5 days

Granulosa cells isolated from immature follicles for *in vitro* culture are neat to circumvent its intrinsic and substantial heterogeneity during *in vivo* growth, thus provide a way to directly assess factors affecting granulosa differentiation.

Note: Prepuberty mice between postnatal days 12–14 are used to isolate immature granulosa cells for culture.

51. Prepare granulosa cells culture medium (see in materials and equipment section).
52. Collect preantral follicles as described in step 40 to step 46.
53. Incubate the collected preantral follicles in 400μL TrypLE Express at 37°C for 10–15 min, and gently pipette to liberate follicular cells (Figure 3D).

54. Stop digestion by adding an equal volume of M2 medium.
55. Spin at 300 × g for 5 min at room temperature and discard the resulting supernatant. Resuspend cells with M2 medium.
56. Filter cell suspension through a 30-μm cell strainer to obtain granulosa cells.
57. Centrifuge the flow-through at 300 × g for 5 min at room temperature and resuspend the pellet in granulosa cells growth medium.
58. Seed cells in a 24-well plate and the plating density is 1×10^6 cells/well. Culture cells in granulosa cells growth medium, allowing cells to adhere for 3 h in an incubator at 37°C containing 5% CO_2.
59. Granulosa cells are starved for 4 h in granulosa cells starvation medium prior to the beginning of experiment treatment. According to what treatment needed, add the substance in granulosa cells starvation medium for further culture (Figure 3D).

EXPECTED OUTCOMES

For the single-cell RNA-seq part, the profile of a successful cDNA sample should have a peak at around 1.5 kb (Figure 4A). The expected average size of final sequencing libraries is around 200–800 bp, with a peak occurs at around 400 bp (Figure 4B). The yield of the library is usually more than 100 ng. The UMAP (Uniform Manifold Approximation and Projection) validates that oocytes, granulosa cells and theca cells enrichment with our method in this protocol (Figure 5).

For the follicle culture part, on day 1 of culture, preantral follicle is composed of an oocyte surrounded by 1–2 layer(s) of granulosa cells, a basal membrane, and some adhering theca cells and/or interstitial cells (Figure 6). By days 6 of culture, in normal conditions, granulosa cells of follicles proliferate and break the basal membrane, the initial *in vivo* organization of follicle is lost while a diffuse or outgrowth pattern is observed (Figure 6).

For the granulosa cell culture part, the purity of isolated granulosa cells should be firstly examined by immunofluorescence analysis by using granulosa-specific markers, for example, Foxl2 (Forkhead box L2) and Cnmd (chondromodulin) (Figure 7). After a designed treatment, effects of the treatment can be evaluated with regard to proliferation, differentiation and steroidogenic capacity of granulosa cells.

LIMITATIONS

This protocol allows to enrich oocytes and capture rare follicular somatic cells to focus on soma-oocyte communications within a follicle. However, an unbiased whole ovary transcriptome cannot be profiled using this protocol, because individual follicles are first isolated to prepare single cell suspension. Further, this protocol is more laborious than whole ovary dissociation followed by high-throughput 10× scRNA-seq. Dissociation of follicles needs to be performed by an experienced experimenter to ensure cell integrity and viability. Pick follicles, single cells, and removal of zona pellucida are also skillful.

TROUBLESHOOTING

Problem 1

Just a few ovulated oocytes are found after superovulation (visible in the before you begin).

Potential solution

Low efficiency of superovulation could be due to several reasons.

Allow newly arrived mice to get acclimated for one week before use, as shipping process, new facility and environment would interfere with several physiological responses. For example, a changed dark-light cycle is known to disturb endogenous LH peak in response to PMSG administration during superovulation.

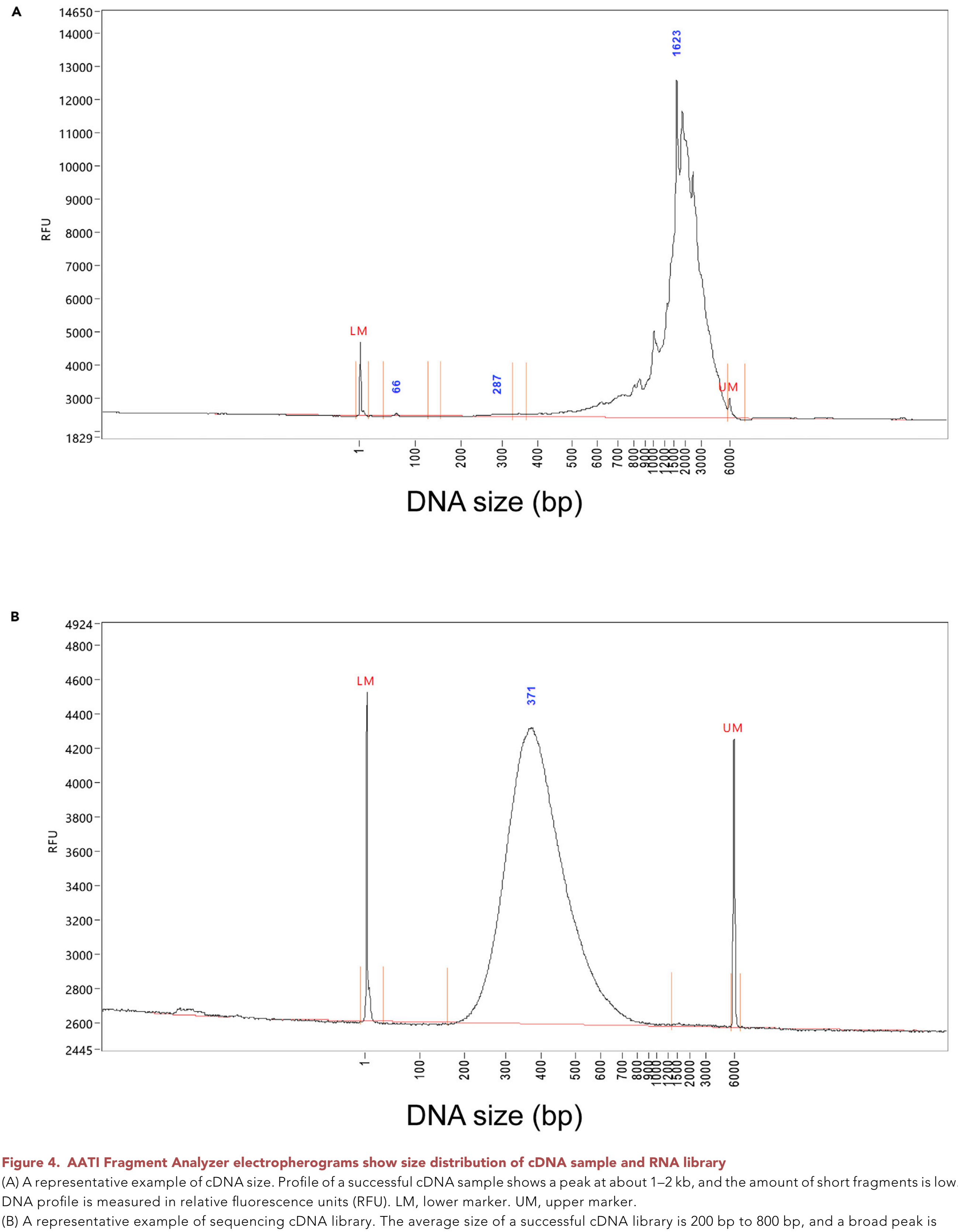


Figure 4. AATI Fragment Analyzer electropherograms show size distribution of cDNA sample and RNA library

(A) A representative example of cDNA size. Profile of a successful cDNA sample shows a peak at about 1–2 kb, and the amount of short fragments is low. DNA profile is measured in relative fluorescence units (RFU). LM, lower marker. UM, upper marker.

(B) A representative example of sequencing cDNA library. The average size of a successful cDNA library is 200 bp to 800 bp, and a broad peak is observed at about 400 bp. DNA profile is measured in relative fluorescence units (RFU). LM, lower marker. UM, upper marker.

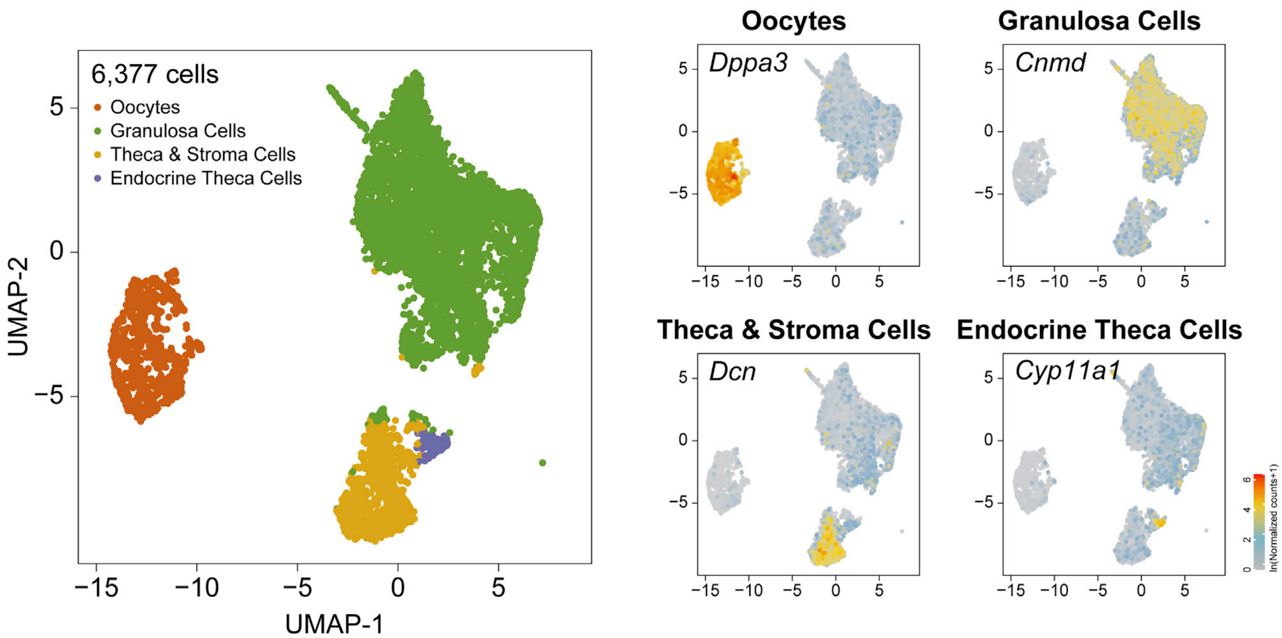


Figure 5. UMAP (Uniform Manifold Approximation and Projection) plots colored by cell type markers

The UMAP projection validates that oocytes, granulosa cells, and theca cells are highly enriched with our method. Adapted with permission from (Long et al., 2022).

Check that the gonadotropins are not expired. Each new batch of gonadotropins should be tested before the beginning of experiments, as efficacy of gonadotropins varies. This can be tested by performing superovulation for fertile females and checking the number of oocytes that can be recovered. In our experience, 15–25 oocytes can be recovered from 6- to 7-week-old C57BL/6J mice.

Dosages of gonadotropins are mostly determined by bodyweights of mice, usually 5 IU of PSMG or hCG is used for 4-week-old mice and 10 IU of PSMG or hCG for 8-week-old mice. Optimization of dosages is also recommended, as possibly there are also strain variations in response to gonadotropins.

Problem 2

Ampulla is not enlarged after superovulation (visible in the section 1: preparation of single metaphase II oocytes and cumulus cells from ovulatory follicles, step 3).

Potential solution

Occasionally ampulla is not swollen 15 h post hCG injection, likely because the cumulus-oocyte complexes start to diffusing. Oocytes can be collected by flushing from infundibulum to utero-tubal junction by using a needle attached to a 1 mL-syringe. Pay attention to gently flush and avoid M2 medium splashing through the narrow utero-tubal junction, which would lose almost all the oocytes. About 0.1 mL prewarmed M2 is needed for each side of oviduct flushing.

Problem 3

Zona-free oocytes stick to a Petri dish (visible in the section 1: preparation of single metaphase II oocytes and cumulus cells from ovulatory follicles, step 8).

Potential solution

Zona-free oocytes sticking to a plastic dish would be difficult to recover without damage. Thus, avoid leaving oocytes reaching bottom of Petri dish by gently pipette up and down, and transfer the

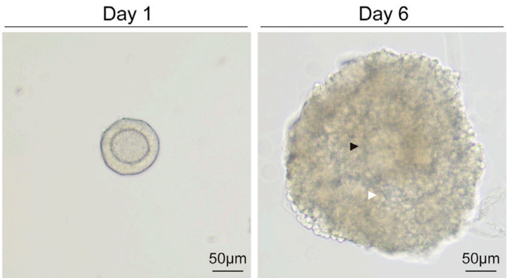


Figure 6. Representative pictures showing morphologies of an isolated preantral follicle at day 1 of culture, and a diffused follicle after 6 days of culture

Black arrowhead indicates the oocyte, white arrowhead indicates onset of an antrum-like cavity. Scale bar, 50 μm.

oocytes into M2 medium quickly after zona disappears. Prolonged exposure in Tyrode's solution impairs oocytes quality and can be lethal in extremely prolonged exposures.

Problem 4

Incomplete zona removal (visible in the section 1: preparation of single metaphase II oocytes and cumulus cells from ovulatory follicles, step 8).

Potential solution

After Tyrode's incubation, oocytes should be checked under a 25 × magnification to determine whether a thin zona remains, as this would compromise efficiency of cell lysis. Incomplete zona removal is mostly caused by carryover of M2 medium that neutralizes Tyrode's. If this is the case, prepare another fresh drop of Tyrode's solution and repeat zona-removal step. Also check that the Tyrode's solution is not expired.

Problem 5

Quality of cDNA sample is poor (visible in the section 3: single-cell RNA-seq library construction, step 32).

Potential solution

The key for RNA-seq is to ensure high RNA quality. A large amount of short fragments in cDNA size distribution profile indicates RNA degradation. To avoid RNA degradation, make sure to set up workbench, tubes, and pipette tips free from RNase and DNase. To ensure cell viability and achieve high-quality mRNA, minimize exposure time of cells in digestive enzymes and promptly pick individual cells into lysis buffer after dissociation. Cells in lysis buffer should be kept on ice until processed to RT.

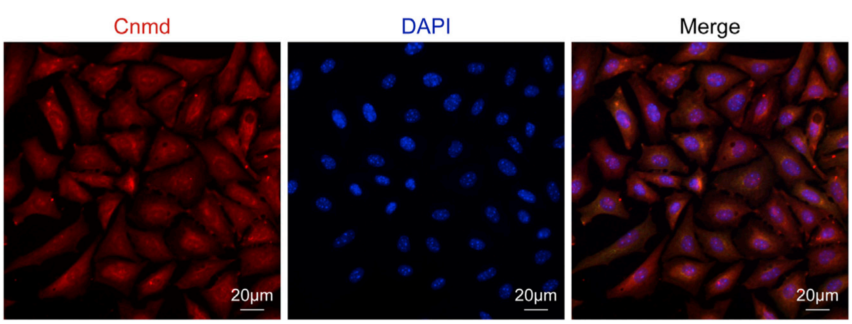


Figure 7. Characterization of *in vitro* cultured granulosa cells

Cultured primary granulosa cells are stained for the granulosa cell marker Cnmd, verifying the purity of isolated cells. Scale bar, 20 μm. Adapted with permission from (Long et al., 2022).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fuo Guo (guofan@ioz.ac.cn).

Materials availability

This study did not generate new material or reagents.

Data and code availability

For detailed analysis and the datasets generated across folliculogenesis, please refer to (Gu et al., 2019; Long et al., 2022).

ACKNOWLEDGMENTS

F.G. is supported by the Ministry of Science and Technology of China (2018YFA0107701), the CAS Project for Young Scientists in Basic Research (YSBR-012), and the Strategic Collaborative Research Program of the Ferring Institute of Reproductive Medicine, Ferring Pharmaceuticals and Chinese Academy of Sciences (FIRMC200510).

AUTHOR CONTRIBUTIONS

F.G. conceived and designed the project. J.Q., R.Z., and R.Y. performed experiments. X.L. performed single-cell analysis. J.Q. and F.G. wrote the manuscript. All the authors approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Andrews, T.S., Kiselev, V.Y., Mccarthy, D., and Hemberg, M. (2021). Tutorial: guidelines for the computational analysis of single-cell RNA sequencing data. Nat. Protoc. *16*, 1–9. https://doi.org/10.1038/s41596-020-00409-w.

Cortvrindt, R., Smitz, J., and Van Steirteghem, A.C. (1996). In-vitro maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepuberal mice in a simplified culture system. Hum. Reprod. *11*, 2656–2666. https://doi.org/10.1093/oxfordjournals.humrep.a019188.

Edson, M.A., Nagaraja, A.K., and Matzuk, M.M. (2009). The mammalian ovary from genesis to revelation. Endocr. Rev. *30*, 624–712. https://doi.org/10.1210/er.2009-0012.

Gu, C., Liu, S., Wu, Q., Zhang, L., and Guo, F. (2019). Integrative single-cell analysis of transcriptome, DNA methylome and chromatin accessibility in mouse oocytes. Cell Res. *29*, 110–123. https://doi.org/10.1038/s41422-018-0125-4.

Lane, M., Robker, R.L., and Robertson, S.A. (2014). Parenting from before conception. Science *345*, 756–760. https://doi.org/10.1126/science.1254400.

Long, X., Yang, Q., Qian, J., Yao, H., Yan, R., Cheng, X., Zhang, Q., Gu, C., Gao, F., Wang, H., et al. (2022). Obesity modulates cell-cell interactions during ovarian folliculogenesis. iScience *25*, 103627. https://doi.org/10.1016/j.isci.2021.103627.

Qian, J., and Guo, F. (2022). De novo programming: establishment of epigenome in mammalian oocytes. Biol. Reprod. ioac091. https://doi.org/10.1093/biolre/ioac091.

Risal, S., Pei, Y., Lu, H., Manti, M., Fornes, R., Pui, H.P., Zhao, Z., Massart, J., Ohlsson, C., Lindgren, E., et al. (2019). Prenatal androgen exposure and transgenerational susceptibility to polycystic ovary syndrome. Nat. Med. *25*, 1894–1904. https://doi.org/10.1038/s41591-019-0666-1.

Saben, J.L., Boudoures, A.L., Asghar, Z., Thompson, A., Drury, A., Zhang, W., Chi, M., Cusumano, A., Scheaffer, S., and Moley, K.H. (2016). Maternal metabolic syndrome programs mitochondrial dysfunction via germline changes across three generations. Cell Rep. *16*, 1–8. https://doi.org/10.1016/j.celrep.2016.05.065.

Simon, L.E., Kumar, T.R., and Duncan, F.E. (2020). In vitro ovarian follicle growth: a comprehensive analysis of key protocol variables. Biol. Reprod. *103*, 455–470. https://doi.org/10.1093/biolre/ioaa073.

Yan, R., Gu, C., You, D., Huang, Z., Qian, J., Yang, Q., Cheng, X., Zhang, L., Wang, H., Wang, P., and Guo, F. (2021). Decoding dynamic epigenetic landscapes in human oocytes using single-cell multi-omics sequencing. Cell Stem Cell *28*, 1641–1656. https://doi.org/10.1016/j.stem.2021.04.012.

Zheng, W., Zhang, H., Gorre, N., Risal, S., Shen, Y., and Liu, K. (2014). Two classes of ovarian primordial follicles exhibit distinct developmental dynamics and physiological functions. Hum. Mol. Genet. *23*, 920–928. https://doi.org/10.1093/hmg/ddt486.