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Isolation and *in vitro* Culture of Mouse Oocytes

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[Abstract] Females are endowed at birth with a fixed reserve of oocytes, which declines both in quantity and quality with advancing age. Understanding the molecular mechanisms regulating oocyte quality is crucial for improving the chances of pregnancy success in fertility clinics. *In vitro* culture systems enable researchers to analyse important molecular and genetic regulators of oocyte maturation and fertilisation. Here, we describe in detail a highly reproducible technique for the isolation and culture of fully grown mouse oocytes. We include the considerations and precautionary measures required for minimising the detrimental effects of *in vitro* culture conditions. This technique forms the starting point for a wide range of experimental approaches such as post-transcriptional gene silencing, immunocytochemistry, Western blotting, high-resolution 4D time-lapse imaging, and *in vitro* fertilization, which are instrumental in dissecting the molecular determinants of oocyte quality. Hence, this protocol serves as a useful, practical guide for any oocyte researcher beginning experiments aimed at investigating important oocyte molecular factors.

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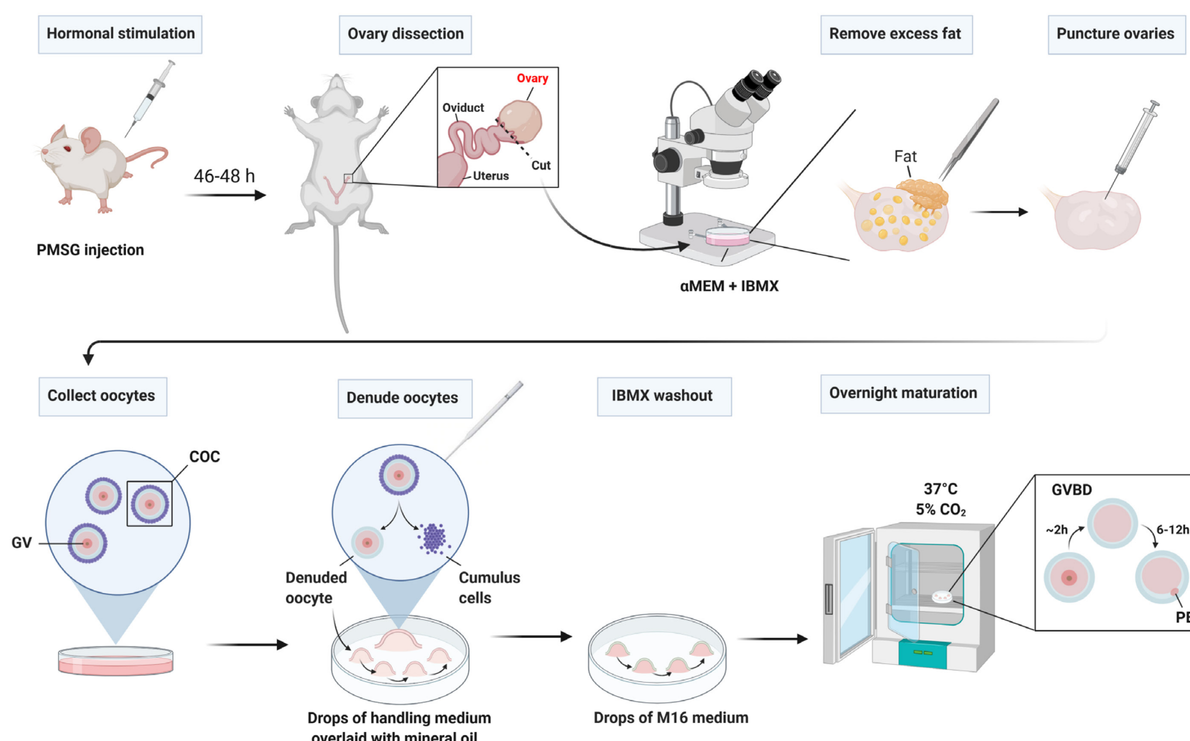
Graphic abstract:



A step-by-step protocol for the isolation and *in vitro* culture of oocytes from mice.

Keywords: Mouse oocytes, Ovary collection, Ovary dissection, Oocyte isolation, Oocyte culture, *In vitro* maturation

[Background] Oocytes provide the overwhelming majority of cytoplasmic building blocks required by the embryo and must undergo two rounds of error-free chromosome segregation to ensure normal embryonic development (Chaigne *et al.*, 2017; Greaney *et al.*, 2017). The first of these divisions, meiosis I (MI), is notoriously error-prone, especially with advancing female age; indeed, errors arising during oocyte MI account for a staggering 80-90% of human aneuploidy (Greaney *et al.*, 2017). MI is also the division in which symmetry-breaking occurs to enable the oocyte to expel half of its chromosomes into a very small daughter cell (known as the polar body, PB), whilst holding on to the bulk of the cytoplasm to support later embryonic development (Chaigne *et al.*, 2017). It is well established that oocyte numbers, ovulation, and oocyte quality are the three oocyte-specific parameters that determine fertility and pregnancy success (Homer, 2020). While there are specific biomarkers and diagnostic tools to test oocyte numbers and ovulation, there is no definitive diagnostic test for oocyte quality. Although human oocytes would be the ideal research material for investigating oocyte quality, extensive restrictions and ethical concerns have made them almost completely inaccessible for experimentation. Hence, the molecular basis of oocyte quality remains poorly understood and will benefit from more in-depth investigation of key aspects such as meiotic maturation, fertilisation, and embryonic development.

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Interrogating the roles of genes that regulate these processes has been accomplished using surrogate mammalian models such as the mouse, which has become the most widely studied model.

Breakthrough advancements in understanding key molecular regulators of MI chromosome segregation and asymmetrical division, as well as the oocyte's vulnerability to aging, have been dependent on the ability to perform detailed analyses of oocytes *in vitro*. The applications of this protocol are wide-ranging and allow for post-transcriptional gene silencing (for example, using RNAi and morpholinos), conventional biochemical analyses such as Western blotting, and in-depth visual interrogation of intracellular structures by immunocytochemistry and time-lapse microscopy. Our laboratory has used all these techniques during *in vitro* culture to uncover numerous unique facets of oocyte regulation, such as spindle assembly checkpoint (SAC) regulation of MI chromosome segregation and its decline with aging (Homer *et al.*, 2005; Gui and Homer, 2012; Riris *et al.*, 2014), a unique oocyte DNA damage response (Subramanian *et al.*, 2020) and new genetic insight into DNA damage-based premature ovarian aging (Subramanian *et al.*, 2021), novel aspects of regulation at the G2-M boundary (Homer *et al.*, 2009; Gui and Homer, 2013), the contribution of late post-anaphase events to asymmetrical division (Wei *et al.*, 2018 and 2020), and key metabolic nodes involved in oocyte aging and potential therapeutics targeting these nodes to reverse poor oocyte quality (Bertoldo *et al.*, 2020; Iljas and Homer, 2020; Iljas *et al.*, 2020).

There are a number of challenges involved in studying oocytes. One major challenge is that oocytes cannot be propagated in the lab like many immortal cell lines and must be isolated as primary cells each time they are required for experimentation. Another challenge is the very long duration of MI, which lasts 6-12 h depending on the mouse strain (versus minutes for mitosis), and requires lengthy experiments. Yet another challenge is the large volume of the oocyte (~40-times larger than that of a somatic cell), which makes the identification of small cellular structures markedly more difficult. It is therefore critically important to develop consistent and reproducible techniques for oocyte isolation and culture.

Oocyte culture methods were first developed as early as the 1930's (Moricard and de Fonbrune, 1937). Later, experiments developed a simple isotonic solution capable of sustaining oocyte maturation (Donahue, 1968 and 1970), which allowed oocytes to be observed during MI for the first time. More sophisticated culture media were subsequently developed (Chatot *et al.*, 1989), and now, oocyte isolation and culture protocols form the basis of all *in vitro* techniques used for studying oocyte biology. Here, we detail a method involving hormonal stimulation and collection of ovaries, followed by isolation and *in vitro* culture of oocytes.

In this protocol, pregnant mare serum gonadotrophin (PMSG) is used to stimulate antral follicle development, thereby increasing the yield of oocytes isolated from the ovary and enabling 30-100 oocytes to be obtained from each mouse depending on the strain and age (Wei *et al.*, 2018 and 2020; Subramanian *et al.*, 2020 and 2021). By minimising exposure to light and temperature fluctuations, this method reduces both the damage caused by *in vitro* culture and the resulting artefacts.

This protocol describes the collection of fully grown oocytes arrested in prophase of MI, characterised by the presence of an intact nucleus, which in oocytes is referred to as the germinal vesicle (GV). Prophase I-arrest can be experimentally maintained using chemicals such as 3-isobutyl-1-

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methylxanthine (IBMX), which sustains high cyclic adenosine monophosphate (cAMP) levels, thereby preventing activation of the major M phase-promoting kinase, cyclin-dependent kinase 1 (Cdk1) (Zhao *et al.*, 2014). Conversely, resumption of meiotic maturation can easily be induced by washing out IBMX, thereby providing a reversible method for manipulating M-phase entry. This approach also allows for the timed introduction of small molecule inhibitors to disrupt regulators at any number of key timepoints during the cell cycle.

The applications of this protocol are extensive and have been critical for advancing our understanding of the regulation of MI.

Materials and Reagents

1. Cell culture dishes; 35 × 10 mm and 60 × 15 mm (Sigma-Aldrich, catalog numbers: CLS430165, CLS 430166)
2. Syringe filter unit; 0.22-µm (Merck, catalog number: SLGP033RS)
3. Microcentrifuge tubes; 0.6-ml and 1.6-ml (Neptune, catalog numbers: 3735.X, 3745.X)
4. Centrifuge tubes; 15-ml and 50-ml (Corning, catalog numbers: 352096, 352070)
5. Disposable ultra-fine needles (27 G × 1/2"; Hanke Sass Wolf, catalog number: 4710004012) and 1-ml syringes (Terumo, catalog number: SS+01T Tuberculin)
6. Glass Pasteur pipettes (230 mm) (VWR, catalog number: 6121702)
7. Mouth aspirator tube assembly (Sigma-Aldrich, catalog number: A5177)
8. Mice (>4 week-old females; Strains used: B6CBF1) (University of Queensland Biological Resources, University of Queensland, Australia)
9. PMSG (Prospec, catalog number: HOR-272)
10. Bovine serum albumin (BSA; Sigma-Aldrich, catalog number: A7096)
11. Light mineral oil suitable for mouse embryo culture (Sigma-Aldrich, catalog number: M5310)
12. IBMX (Sigma-Aldrich, catalog number: I5879)
13. MilliQ water
14. Sodium bicarbonate (Sigma-Aldrich, catalog number: S5761)
15. HEPES (Free acid) (Sigma-Aldrich, catalog number: H3375)
16. HEPES (Salt) (Sigma-Aldrich, catalog number: H7006)
17. Gentamicin sulfate (Sigma-Aldrich, catalog number: G1264)
18. M16 medium (Sigma-Aldrich, catalog number: M7292) for *in vitro* culture
19. α-Minimum Essential Medium (MEM) powder (Thermo Fisher Scientific, catalog number: 12000022) (see Recipes)

Equipment

1. Fine dissecting scissors (Met-App Scientific and Surgical Instruments, catalog number: 2235)
2. Fine forceps (Met-App Scientific and Surgical Instruments, catalog number: 2127)

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3. Stereomicroscope (Leica Microsystems, model: M165C; Nikon Microscope Solutions, model: SMZ800N)
4. Dry heating block (Grant Instruments, model: QBD4)
5. CO_2 incubator (Sanyo, model: MCO-18AIC)
6. Alcohol burner (LabTek, model: LW15557-01)
7. Tube roller mixer (Ratek, model: BTR10P-12V)
8. Magnetic stirrer (IKA, model: RCT basic)

Procedure

A. Hormonal stimulation of mice

1. Prepare 20 IU/ml PMSG in saline and aliquot 1-ml volumes into 1.6-ml microcentrifuge tubes. Store at -80°C until required (stored aliquots are stable up to 12 months).
2. Inject female mice intraperitoneally at the lower left-hand side of the abdomen with approximately 300 µl PMSG to a final dosage of 5-7 IU.
3. House mice in sterile filter-top cages at 25°C for 46-48 h and feed a standard diet and water.

B. Preparation of the mouth aspiration unit

1. Light the alcohol burner containing absolute ethanol.
2. Take a glass Pasteur pipette with the ends held between the thumb and forefinger of each hand and hold the narrow end over the flame so that it begins to soften.
3. Remove the pipette from the flame and rapidly pull each end apart so that the softened glass can be stretched with the goal of achieving an internal diameter similar to that of an oocyte (approximately 80 µm). This may require numerous attempts to achieve the correct diameter, which can be ascertained by comparing the size of the pulled tip to that of oocytes after isolation.
4. Cut or break the narrow length of the pipette to an appropriate length for handling.
5. To assemble the mouth aspiration unit, attach the Pasteur pipette to a 1,000-µl volume pipette tip, then place the tip into a mouth aspiration tube as pictured in Figure 1Aii.

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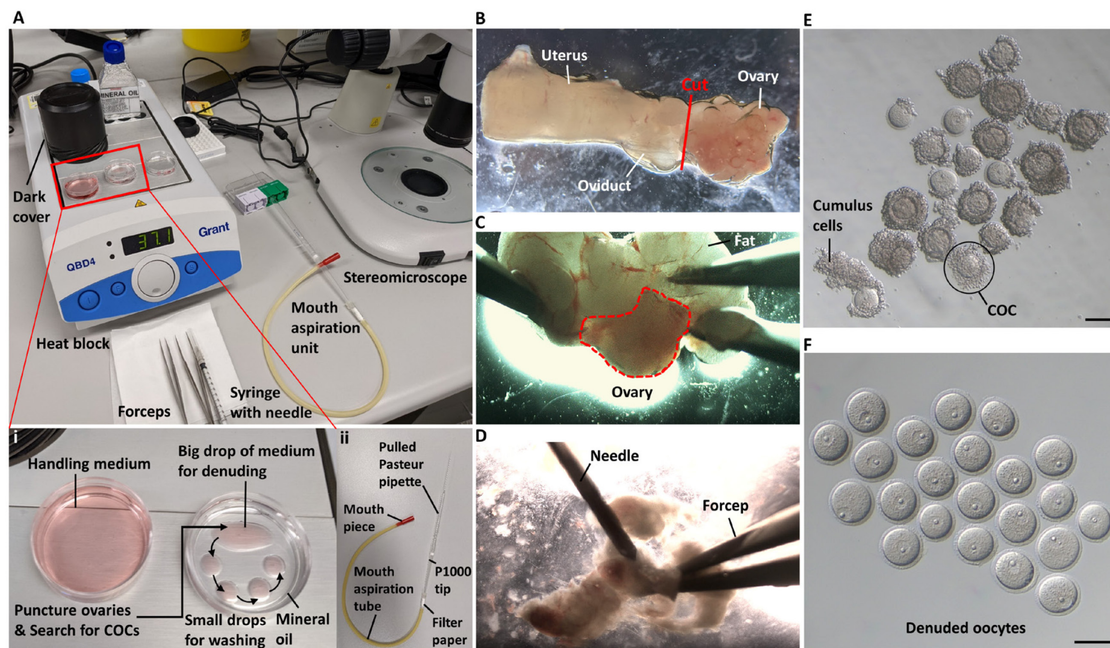

Figure 1. Workflow for ovary collection and oocyte isolation. (A) Preparation of the dissection station. The insets depict dishes for isolating, denuding, and serial washing of oocytes (i) and the mouth aspiration unit assembly (ii). (B) Enlarged brightfield images of a mouse ovary with attached oviduct and uterine horns. (C) The ovary is dissected at the boundary between the ovary and the oviduct (cut site). Shown is an enlarged image of an ovary enclosed in fat. (D) Example of an ovary (fat removed) being disaggregated to release cumulus-oocyte complexes (COCs). (E) Isolated COCs. (F) Denuded oocytes following removal of cumulus cells. Scale bar: 50 µm.

C. Preparation of the handling and culture media

1. On the day before ovary collection, filter 5 ml M16 culture medium through a 0.22-µm syringe-driven filter into a 15-ml centrifuge tube and place the tube with its lid loosened in an incubator set to 5% CO_2 and 37°C to allow for CO_2 equilibration (see Note 1).
2. On the day of ovary collection (46-48 h following PMSG injection), prepare 3-4 100-µl droplets of CO_2-equilibrated M16 in a 35-mm culture dish and overlay with mineral oil to prevent evaporation. This dish will be used for overnight culture of oocytes post-collection.
3. Decant 5 ml handling medium into a 15-ml centrifuge tube and add 0.3% BSA w/v (5 ml medium requires 15 mg BSA).
4. Place the handling medium on a tube roller mixer with the tube lid tightly closed to allow the BSA to dissolve (approximately 15 min).
5. Filter the handling medium through a 0.22-µm syringe-driven filter, add IBMX to a final concentration of 100 µM (see Note 2), and pre-warm to 37°C by placing on a dry heating block.
6. Prepare the workstation with the equipment pictured in Figure 1A.
7. Prepare droplets of IBMX-treated handling medium in a 35-mm culture dish overlaid with mineral oil, as pictured in Figure 1A inset.

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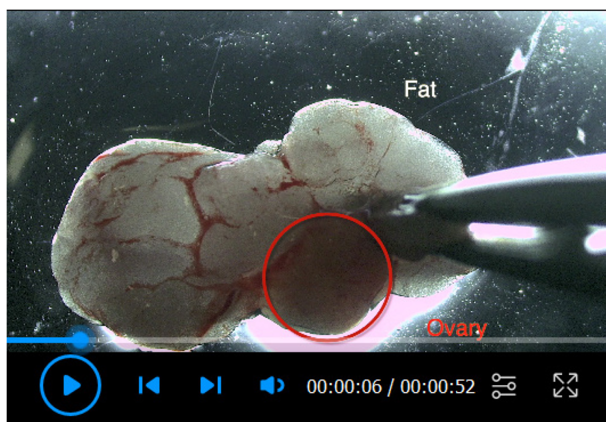
8. Prepare 1 ml IBMX-treated handling medium in a 1.6-ml microcentrifuge tube and pre-warm to 37°C on a dry heating block.

D. Ovary collection

1. Sacrifice the mouse by cervical dislocation and place in the supine position. Spray the abdomen with 70% ethanol to wet the fur and prevent it from entering the abdominal cavity.
2. Make a small incision in the skin on the lower abdomen with fine dissecting scissors and use gloved hands to gently pull the abdominal skin open to expose the peritoneal membrane.
3. Clean any fur from the scissors with a paper towel, then cut the peritoneal membrane to expose the abdominal cavity.
4. Using fine forceps, locate the uterine horn. Gently pull on the uterine horn, follow it cephalad to its extremity, and locate the ovary just inferior to the lower pole of the kidney.
5. Use fine dissecting scissors to transect a portion of the uterine horn adjacent to the ovary and dissect the para-ovarian fat from surrounding tissues, as pictured in Figure 1B and C.
6. Place the ovaries in the tube containing 1 ml pre-warmed IBMX-treated handling medium prepared in Part C and transfer to the lab.

E. Oocyte isolation

1. In the lab, transfer ovaries into a 35-mm culture dish.
2. Under a stereomicroscope, use fine forceps and the tip of a 27 G needle attached to a 1-ml syringe to carefully remove the surrounding oviduct, uterine horn, and fat from the ovaries, ensuring that the ovary is not damaged, as shown in Figure 1C and Video 1. The ovary appears light-pink in colour, while fat and the oviduct are white in colour.



Video 1. Isolation of oocytes from a mouse ovary

3. Once the ovaries are free of fat and other tissue, transfer them into another 35-mm culture dish containing 2 ml handling medium. Do not overlay this medium with mineral oil.
4. Puncture the ovaries repeatedly with the needle to release cumulus-oocyte complexes (COCs), as pictured in Figure 1D and Video 1.

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5. Using the pulled glass Pasteur pipette prepared in Part B, select COCs (Figure 1E) from the dish and move to a second 35-mm culture dish with IBMX-treated handling medium droplets overlaid with mineral oil (Figure 1Ai).
6. Denude oocytes of surrounding cumulus cells using the mouth-controlled aspiration unit to repeatedly aspirate and expel COCs through the tip of the glass pipette to produce cumulus-free oocytes, as pictured in Figure 1F and Video 1 (see Note 3).
7. Wash the denuded oocytes with sequential droplets of IBMX-treated handling medium to remove any residual cumulus cells.

F. Oocyte culture

1. To induce resumption of meiosis, wash denuded oocytes with 5-6 100-µl droplets of IBMX-free M16 in a 60-mm culture dish (no mineral oil overlay).
2. Place oocytes in the culture dish prepared in Part C (Figure 2A) and place in an incubator at 37°C and 5% CO_2 for overnight culture.
3. For analysing the rates of GV breakdown (GVBD), remove oocytes from the incubator 2 h following washout from IBMX and score for GVBD under a light microscope by checking for the disappearance of the GV (as pictured in Figure 2B).
4. Rates of polar body extrusion (PBE) can be scored approximately 8-10 h following release from IBMX by checking for the presence of a PB under a light microscope, as pictured in Figure 2C. For time-series data, as shown in Figure 2D and E, score for GVBD and PBE at 30-min intervals (see Notes 4 and 5).

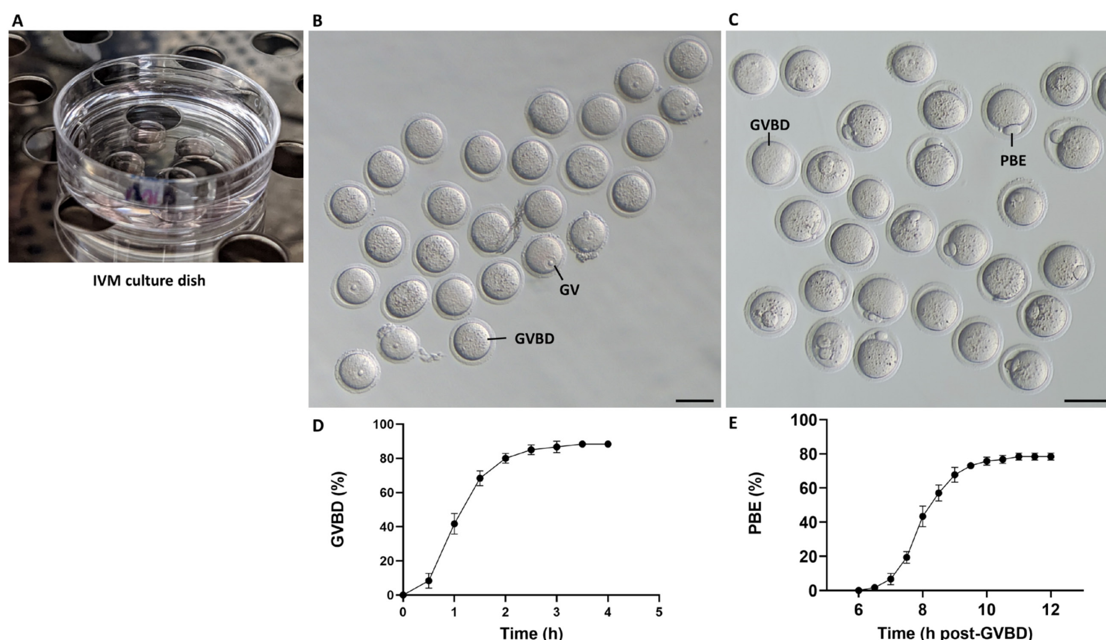

Figure 2. Workflow for the *in vitro* maturation (IVM) of oocytes. (A) A culture dish containing drops of M16 medium under mineral oil overlay in the incubator used for IVM. (B) Brightfield image of oocytes cultured in IBMX-free M16 medium. Note that after 2 h, most oocytes have

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undergone GVBD. (C) Following overnight culture in IBMX-free M16 medium, a small PB has been extruded by the majority of oocytes. (D, E) GVBD and PBE rates in relation to time post-release from IBMX and post-GVBD, respectively. Error bars represent the mean of three biological replicates ± standard error of the mean (SEM). Scale bar: 50 μm.

Data analysis

1. Calculate the proportion of oocytes undergoing GVBD as the ratio (%) of GVBD oocytes to the total number of oocytes. Similarly, calculate PBE rates as the ratio (%) of oocytes that have extruded a PB to the total number of oocytes.
2. Repeat scoring at the desired time intervals for the desired total duration of time. For instance, GVBD rates may be calculated at 0.5-h intervals for a total of 4 h post-IBMX release, as in Figure 2D. PBE rates may be calculated at 0.5-h intervals from 6-12 h post-GVBD, as in Figure 2E (see Notes 5 and 6).
3. After obtaining data from ≥ 3 biological replicates, use GraphPad Prism (www.graphpad.com) to plot the mean percentages on a line graph, with time intervals on the x-axis and respective percentages on the y-axis.

Notes

1. M16 medium requires CO_2 equilibration before use. As M16 contains Phenol red pH indicator, this can provide indication of whether it is appropriately equilibrated. Medium that appears orange-red is appropriately equilibrated, but medium that is pink in appearance has not been equilibrated for long enough and should not be used for oocyte culture.
2. IBMX is added to inhibit GVBD during oocyte isolation and handling stages. IBMX may also be added to the M16 culture medium to maintain oocytes in the GV stage for a longer period of time.
3. Minimise light exposure to the oocytes by covering the handling and culture dishes as much as possible with a dark cover, as pictured in Figure 1A.
4. Removing the culture dish from the incubator frequently can expose the oocytes to temperature fluctuations and excessive light, which may affect maturation rates.
5. The oocyte yield and timing of GVBD and PBE can vary depending on the mouse strain (Parker-Thornburg *et al*., 2011; Polanski, 1986 and 1997; Cheng *et al*., 2012). Researchers are encouraged to first evaluate a few strains to identify the one that best suits their needs (Luo *et al*., 2011).
6. Time-lapse microscopy was employed in our laboratory to automatically capture brightfield images at desired intervals. If manually scoring PBE on the following day of IVM, the PB may be degraded and difficult to identify.

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7. Use only clean and sterile beakers, volumetric flasks, weighing trays, etc., for making the handling medium.
8. It is important to maintain the pH of the medium between 7.2 and 7.5. If the pH does not stabilise around this range, re-make the medium.
9. The addition of HEPES to the handling medium allows use outside of a CO_2 incubator without significant pH fluctuation.

Recipes

1. Oocyte handling medium
   Final concentration: 6 mM sodium bicarbonate, 10 mM HEPES (free acid), 10 mM HEPES (sodium salt), 100 pg/ml gentamicin sulfate. See Notes 7-9.
   a. Weigh 8.8 g α-MEM powder in a weighing tray and transfer to a 1-L glass beaker.
   b. Add 900 ml MilliQ water and stir on a magnetic stirrer.
   c. Add 504 mg sodium bicarbonate, 2,380 mg HEPES (free acid), and 2,600 mg HEPES (sodium salt) to the beaker and stir for at least 15 min at room temperature.
   d. Once the solutes are completely dissolved, check the pH of the handling medium under constant stirring. Ensure that the pH of the medium is 7.2-7.5.
   e. Make the volume up to 1 L, filter the medium into sterile glass bottles, label, and store at 4°C for no more than 2 months.
   f. On the day of the experiment, decant the required volume of handling medium into centrifuge tubes and add 0.3% w/v BSA and 100 μM IBMX immediately before starting the experiment. As an example, for 10 ml media, add 30 mg BSA and 5 μl IBMX (stock concentration: 200 μM).

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This protocol was adapted with minor modification from a previous study published by Subramanian *et al*. (2020).

Competing interests

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H.A.H. is a co-founder, shareholder, and advisor of Jumpstart Fertility Inc., which was founded to develop research into NAD$^+$-dependent pathways involved in female fertility. H.A.H. is a co-inventor on the patent application 'Methods for increasing fertility' (application number 2017903013). All remaining authors declare no competing interests.

Ethics

All mice used in the preparation of this protocol were approved by the University of Queensland Office of Research Ethics, Animal Ethics Committee under certification number MED/530/18.

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