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Ex vivo imaging of ovulation in mouse ovarian follicles

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We use this protocol and it's working

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Abstract

Understanding ovarian function requires a clear and detailed picture of biological processes that take place deep inside the body, within a narrow window of time. Essential progress is being held back by the limited experimental approaches currently available to study the ovarian cycle in real time. We recently developed a model system to study ovulation live using high-resolution microscopy. We adapted a long-term membrane-based culture system of isolated mouse follicles for use with advanced quantitative live imaging. In brief, antral follicles are mechanically isolated from whole mouse ovaries and cultured in medium containing hormone combinations that mimic late folliculogenesis and ovulation. This enabled us to, for the first time, resolve the entire 12-hour-long ovulatory process at cellular resolution. To follow the behaviour of individual cells, we used transgenic mice expressing a membrane (Myr-tdTomato) and a histone (H2B-GFP) marker. Using confocal microscopy, we were able to follow the dynamic behaviour of cells and the oocyte inside the follicle. We were also able to use two-photon microscopy and 3D reconstructions to measure follicle volume and shape changes during ovulation. This generated robust quantitative measurements of the process. The major advantage of our method is the introduction of multiple imaging modalities and perturbations, allowing for the detailed visualisation and manipulation of ovarian function in real time. In this protocol, we describe the procedures for follicle isolation, culture, imaging.

Isolation, culture and imaging of mouse ovarian follicles for the study of ovulation

1 Prepare 10 ml of isolation medium and 10 ml of culture medium:

Isolation medium
9.4 ml Alpha-MEM (1X) + GlutaMAX + HEPES (Thermo Fisher, 42360032)
100 µl 100 IU/ml Penicillin-Streptomycin (Gibco, 15140122)
500 µl FBS (Gibco, 16000-044)

Culture medium
9.3 ml Powdered alpha-MEM (Thermo Fisher, 12000014)
100 µl 100 IU/ml Penicillin-Streptomycin (Gibco, 15140122)
500 µl FBS (Gibco, 16000-044)
100 µl 10x ITS-G (Gibco, 41400-045)

2 Mix well and filter sterilise using a 0.22 µm sterile filter and plastic syringe.

3 For both the isolation medium and the culture medium, move 4.5 ml into a fresh 15 ml tube and supplement with 15 µl of 10 µg/ml FSH (National Hormone and Peptide Programme #NIDKK-oFSH-20 (ovine FSH; final concentration 33 ng/ml)). The remaining 5 ml of each medium without FSH can be left overnight in a 37°C, 5% CO2, 20% O2 incubator for use in a later step.

4 Pipette 4 ml of isolation medium + FSH into two 35 mm plastic isolation dishes (2 ml per dish) and 100 µl into an eppendorf tube.

5 Pipette 1.6 ml of culture medium + FSH into each of two wells of a 6-well culture plate (Corning 3516) and insert a Millicell 30 mm diameter, hydrophilic PTFE membrane, 0.4 µm pore size cell culture insert (Millipore, PICM03050) above the medium in each well.

6 Allow the isolation dishes, eppendorf tube, and 6-well culture plate to equilibrate in a 37°C, 5% CO2, 20% O2 incubator for 30 minutes prior to starting the isolation.

7 Excise ovaries from female mice aged 23-28 days using scissors and forceps. These mice are pre-pubertal and contain a large number antral follicles. Since they have never ovulated, the ovaries do not contain corpora lutea, which facilitates easy isolation of antral follicles.

8 Collect ovaries in the eppendorf tube containing 100 µl isolation medium.

9 Transfer ovaries into a 35 mm plastic dish containing 2 ml of isolation medium. A typical live imaging experiment uses two mice.

10 Isolate large antral follicles greater than 300 µm in diameter from the ovaries using 30G x ½" hypodermic needles (BD Microlance 304000) attached to plastic syringes on a Zeiss SteREO Discovery V8 stereoscope with a heated platform at 37°C. The follicles are first isolated from the rest of the ovarian tissue and then cleaned to remove as much of the surrounding tissue as possible without disturbing the theca layer of the follicle itself.

11 The isolated and cleaned follicles are then transferred to the 6-well culture plate containing culture medium onto the cell culture insert using a mouth pipette and 750 µm diameter glass capillary (Hilgenberg, 1411012). The follicles should be evenly spaced, each in a separate droplet, with approximately 20 follicles per insert.

12 The follicles are then cultured in a 37°C, 5% CO2, 20% O2 incubator for 24 hours. This initial culture period in the presence of FSH stimulates LH receptor expression, which is crucial for them to be able to respond to the ovulatory stimulus. To mimic the ovulatory stimulus in our system, we use human chorionic gonadotrophin (hCG), which binds the LH receptor to trigger ovulation.

13 In a typical experiment, we may want to compare the effect of the drug-based perturbation of a protein of interest on ovulation. For drug treatments, healthy follicles should be selected from the 6-well culture plate from the previous day after 22-26 hours of culture and moved with a mouth pipette and glass capillary into a 4-well plastic Nunc plate (Thermo Fisher, 176740) containing culture medium supplemented with DMSO/drug and pre-incubated for 1 hour in a 37°C, 5% CO2, 20% O2 incubator.

14 An imaging setup is then constructed using a rectangular Ibidi glass-bottomed 2-well µ-Slide (80287) and two Millicell 12 mm diameter, hydrophilic PTFE membrane, 0.4 µm pore size cell culture inserts (Millipore, PICM01250). Using scissors, remove the plastic feet from the inserts, such that the plastic outer ring at the base of the insert is flat. The inserts are then fixed to the glass bottom of the imaging dish using 3x SecureSeal 120 µm adhesive imaging spacers (Grace Bio-Labs, 654002) per membrane insert, cut into small squares of around 2 mm x 2 mm.

15 1.4 ml of ovulation medium is then added to each well, supplemented with DMSO/drug if necessary, taking care to pipette the medium underneath the membrane. Ovulation medium can be prepared by taking 4.5ml of the culture medium prepared on the previous day and supplementing with 15 µl of 10 µg/ml FSH (National Hormone and Peptide Programme #NIDKK-oFSH-20 (ovine FSH; final concentration 33 ng/ml)), 22.5 µl of 1.6 mg/ml hCG (Ovogest, A211A05; final concentration 8µg/ml) and 2.25 µl of 8 µg/ml EGF (Roche, 52779000; final concentration 5 ng/ml).

16 Allow the imaging setup to equilibrate in a 37°C, 5% CO2, 20% O2 incubator for 30 minutes prior to transferring the follicles.

17 Following pre-incubation with DMSO/drug, the follicles should be moved from the 4-well plastic Nunc plate onto the membraneinserts of the imaging setup using a mouth pipette and glass capillary.

18 The dish is then transferred to the microscope for imaging as quickly as possible.

Imaging experiments are performed on Zeiss LSM800 and LSM980 (or other) inverted confocal microscopes equipped with a long working distance objective to allow imaging deep into the follicle (Zeiss LD LCI Plan-Apochromat 25x/0.8 Imm Corr DIC M27 (NA = 0.8) (FWD = 0.57 mm at CG = 0.17 mm) for confocal imaging or Zeiss Plan-Apochromat 20x/0.8 M27 (NA = 0.8) (FWD = 0.57 mm at CG = 0.17 mm for two-photon imaging)). The imaging chamber is heated to maintain the temperature of the imaging medium at 37.5°C and is additionally supplied with 5.5% CO2 and wet tissue to ensure sufficient humidity in the surrounding air to prevent evaporation. A combined approach of confocal and two-photon microscopy allowed us to describe detailed cell movements, oocyte movement in 3D and volumetric changes of the whole follicle during ovulation. This experimental system allowed us to image ovarian follicles for >24 hours, visualising the entire process of ovulation. Ovulation was successful in >90% of the follicles imaged.