

## Video Article

# Culture and Co-Culture of Mouse Ovaries and Ovarian Follicles

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## Abstract

The mammalian ovary is composed of ovarian follicles, each follicle consisting of a single oocyte surrounded by somatic granulosa cells, enclosed together within a basement membrane. A finite pool of follicles is laid down during embryonic development, when oocytes in meiotic arrest form a close association with flattened granulosa cells, forming primordial follicles. By or shortly after birth, mammalian ovaries contain their lifetime's supply of primordial follicles, from which point onwards there is a steady release of follicles into the growing follicular pool.

The ovary is particularly amenable to development *in vitro*, with follicles growing in a highly physiological manner in culture. This work describes the culture of whole neonatal ovaries containing primordial follicles, and the culture of individual ovarian follicles, a method which can support the development of follicles from an immature through to the preovulatory stage, after which their oocytes are able to undergo fertilization *in vitro*. The work outlined here uses culture systems to determine how the ovary is affected by exposure to external compounds. We also describe a co-culture system, which allows investigation of the interactions that occur between growing follicles and the non-growing pool of primordial follicles.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/52458/>

## Introduction

The mammalian ovary is composed of a female's lifetime supply of oocytes, each contained within an ovarian follicle. The follicles are formed prior to birth at the resting, primordial stage: once the follicle pool is established, there is a continual and gradual movement of follicles from the primordial into the growing follicle pool. As follicles start to grow, they develop through the primary, secondary, preantral and then antral stages, until they reach the preovulatory, or Graafian, stage. Only oocytes from preovulatory follicles have full developmental competence, able to support embryonic development to term, if fertilized.

The ovary has long been known to develop in a highly physiological manner *in vitro*. This is likely to be due to each ovarian follicle containing within it the cells necessary to support an oocyte from the immature stage (at which point it is unable to complete its meiotic division) through to the developmentally competent stage (at which point it can fully support the completion of meiosis, fertilization and the development of the resulting embryo to term).

The physiological nature of ovary development *in vitro* has led to the wide use of ovary culture techniques. Consequently, *in vitro* methods have been used to investigate the regulation of ovary development, ovarian pathology (for instance that of polycystic ovarian syndrome, PCOS), examination of how ovaries/oocytes are affected by exposure to chemicals, and also with the practical aim of obtaining fertilizable oocytes from primordial ovarian follicles. To date, the latter has been achieved only using mouse ovarian tissue<sup>1</sup>, although culture techniques using follicles from large mammals, including humans, have greatly improved in recent years<sup>2,3</sup>.

We describe here several culture methods using mouse ovarian tissue. The first method uses whole neonatal mouse ovaries, and supports the formation and early development of primordial follicles<sup>4</sup>. The second system supports the growth of individual, intact ovarian follicles from the late preantral to the preovulatory stage; using this technique, follicles can be cultured individually, or in pairs<sup>5,6</sup>. Finally, we describe a co-culture system, combining the first two techniques in a method that allows investigation of the interactions between growing and primordial ovarian follicles<sup>7</sup>.

Culture of individual intact ovarian follicles allows follicle growth to be determined from daily follicle measurements during the culture period, while medium analysis allows investigation of follicle/ovary hormone production. Further tissue analyses can be achieved by a collection of follicles or ovarian tissue at the end of culture, for histological/immunohistological analyses or for subsequent processing for example to obtain mRNA or proteins.

## Protocol

All animal work was performed in accordance with institutional guidelines under license by the UK Home Office (project license number PPL 60/1726 and 60/4026).

Note: House animals in accordance with UK legal requirements in a 14 hr light and 10 hr dark photoperiod. Conduct experiments on animals using wild type C57Bl6J mice, Tau-GFP mice that have ubiquitous expression of green fluorescent protein (GFP)<sup>8</sup>, and Thy1-YFP mice with occasional expression of yellow fluorescent protein (YFP) in a subset of neuronal cells<sup>9</sup>: both transgenic lines were bred on a C57Bl6J background.

### 1. Working Conditions and Preparation of Instruments

1. Perform all media preparation, tissue dissections, and culture work in a laminar flow hood to ensure sterility: this avoids the requirement of the addition of antibiotics to media.
2. Always allow media/culture plates/embryo dishes to equilibrate for at least 1 hr, in a 37 °C oven (dissection medium/embryo dishes) or 37 °C, 5% CO<sub>2</sub> incubator (culture medium and plates), prior to use.
3. Soak glass pipettes in 0.1% solution of bovine serum albumin (BSA) for around an hour, and then leave to dry. Pull pipettes in the flame of a Bunsen burner, bending the glass as you do so, to produce finely drawn curved glass pipettes. After the pipette is pulled, make a clean cut with a glass cutter. Oven sterilize at 160 °C for around 45 min.  
NOTE: A store of these glass pipettes will be needed to transfer ovaries and follicles.

### 2. Preparation of Dissection and Culture Media

1. Prepare Dissection Medium.
  1. Dissolve 3 mg/ml BSA in Leibowitz L15 medium and filter sterilize through a 0.2 µm pore, 25 mm diameter filter.
2. Neonatal Ovary Culture.
  1. To prepare medium for neonatal ovary culture, make 1 ml of medium for each ovary that will be cultured. Dissolve 3 mg/ml BSA in  $\alpha$ -Minimal Essential Media ( $\alpha$ MEM) and filter sterilize through a 0.2 µm pore, 13 mm diameter filter into a sterile tube.
  2. To prepare plates for neonatal ovary culture, add 1 ml of neonatal ovary culture medium into each well (one ovary per well) of a 24-well plate. Using sterile watchmaker forceps, place one polycarbonate membrane on top of the medium in each well, shiny surface up. UV sterilize membranes before use.
3. Follicle Culture.
  1. To prepare medium for follicle culture, supplement  $\alpha$ -Minimal Essential Media with 1 IU/ml recombinant human follicle stimulating hormone, 5 µg/ml ascorbic acid and 5% v/v serum obtained from adult female mice. Filter sterilize through a 0.2 µm pore, 13 mm diameter filter into a sterile tube.
  2. Filter sterilize silicon oil through a 0.45 µm pore, 25 mm diameter filter, into a sterile tube.
  3. To prepare plates, place 30 µl droplets of follicle culture medium into each well of a non-tissue culture treated 96-well microtiter round-well plate, using only the top row of each plate (to allow follicles to be moved into new rows each day over the culture period). Carefully overlay medium with 70 µl of sterilized silicon oil, to prevent medium evaporation.
4. Follicle-ovary Co-culture.
  1. Prepare medium for follicle-ovary co-culture as for follicle culture in step 2.3.1 above, making up 1 ml of medium for each follicle-ovary co-culture being set up.
  2. To prepare plates, add 1 ml of medium into each well of a 24-well plate. Using sterile watchmaker forceps, place one Nucleopore membrane on top of the medium in each well, shiny surface up. UV sterilise membranes before use.

### 3. Neonatal Ovary Dissection and Culture

1. Place 1 ml of dissection medium into each sterile glass embryo dish.
2. Cull neonatal mouse pups aged between postnatal day 0 and 5, culling by decapitation according to UK Home Office regulations.
3. Grasp the skin covering the abdominal wall using a pair of fine dissection forceps and make a large incision in the skin and body wall. Pull open the incision so the entire abdomen is exposed. The bladder is usually engorged at this stage and can be punctured to make dissection easier.
4. Move the guts out of the way using watchmaker forceps. Follow the uterine horns from the bladder up to the kidney on each side. The ovary is located just below the kidney at the top of the uterus and will appear as a cloud-like structure under a dissecting microscope.
5. Grasp the ovary gently with watchmaker forceps, and use scissors to sever its attachment to the uterus. Transfer the pair of ovaries into embryo dishes containing pre-warmed dissection medium.
6. Carry out fine dissection of ovaries under a dissection microscope on a heated stage (37 °C). Use insulin needles to trim away the bursal sac and any excess material including the fallopian tube, until only the ovary remains.
7. Transfer each ovary into the well of a culture plate prepared in step 2.2.2 above, using a finely drawn curved glass pipette, one ovary on top of each membrane (see **Figure 1A**). Culture in a 37 °C, 5% CO<sub>2</sub> incubator.
8. Change the medium every second day. Use a pipette to exchange 50% of the medium in each well for pre-gassed fresh medium: place the pipette tip at the edge of the well of medium to avoid disturbing the membrane.
9. Maintain cultures for up to 6 days.

10. At the end of the culture, freeze medium and fix or freeze ovaries after a brief wash in PBS, as required.
  1. Fix ovaries in Bouin's fixative for 90 min for histological analysis, or in 10% neutral buffered formalin for 90 min for immunohistological analysis. Snap freeze ovaries by placing tissue in a polypropylene tube, placing the tube in dry ice for 5-10 min before freezing at -70 °C for subsequent protein or mRNA extraction.

## 4. Follicle Dissection and Culture

1. Cull 19-23 day old female mice and isolate the ovaries. Wet the fur with 70% ethanol before making any incisions. Pinch the skin using blunt ended forceps and make a large incision in the abdomen with dissection scissors, penetrating through both the skin and the body wall.
2. Move the gut out of the way using a finer set of dissection instruments and locate the uterus. Follow the uterus up to the ovary which is located below the kidney on each side.
3. Gently grasping the ovary using a pair of watchmaker forceps, sever the ovaries attachment to the uterus with fine dissection scissors. Avoid collecting too much of the ovarian fat pad. Collect the ovaries and transfer into an embryo dishes containing pre-warmed dissection medium.
4. Remove the ovarian bursa using insulin needles to trim away the bursal sac and any excess material including the fallopian tube, until only the ovary remains. Roughly halve each ovary using insulin needles.
5. Carefully transfer each ovary-half into an individual watch glass containing 1 ml dissection medium. Cover each watch glass with a glass slide to prevent evaporation of medium and to ensure sterility.
6. Store watch glasses containing ovary halves in a 37 °C oven until needed, but carry out the next dissection step as soon as possible. Discard tissue stored in this way for more than an hour.
7. Transfer watch glass containing an ovary half to a 37 °C heated microscope stage in a laminar flow hood. Roughly dissect ovary into large pieces using two insulin needles, in order to identify late pre-antral follicles as follows: these will contain 2-3 layers of granulosa cells and have a diameter of around 180-200 µm.
8. Manually dissect out any identified follicles using one insulin needle and one 30 x 0.25 mm acupuncture needle that has been secured in a needle holder. Be careful to remove most of the surrounding stroma, but avoid damaging the basal lamina of follicles (see **Figure 1B**).
9. Use a finely drawn curved glass pipette to carefully transfer dissected follicles into a collecting watch glass containing pre-warmed dissection medium. Be careful to keep follicles within the thin caliber section of pipette to avoid losing follicles.
10. Measure follicles accurately using a calibrated eyepiece graticule fitted into a dissecting microscope.
11. Select follicles for culture only if they measure  $190 \pm 10$  µm in diameter. Further select only healthy, spherical follicles for culture; these will be translucent, without dark atretic areas, and have an intact basal lamina, along with some attached thecal tissue: discard any follicles that do not fit this description. A yield of between 10-15 follicles per ovary is good.
12. Use a finely drawn curved glass pipette to transfer a single follicle into the well of a plate made up as in step 2.3.3 above. Carefully place the follicle in the bottom of the well (and not in the upper oil layer). Culture in a 37 °C, 5% CO<sub>2</sub> incubator.
13. Culture follicles for up to 6 days, moving follicles into a well containing fresh medium every day.
  1. Prepare the next row in the 96-well plate as in plate preparation in step 2.3.3 above. Return plate to the incubator for at least an hour, to allow equilibration. Transfer follicles into fresh wells of medium, using a finely drawn glass pipette.
  2. If at any point in the culture follicles are to be left for two days before moving into fresh medium, place each follicle in a minimum of 60 µl (rather than 30 µl) droplets of follicle culture medium.
14. To collect data on follicle growth, measure follicles daily, using an eyepiece graticule fitted into a dissecting microscope. The oil layer will distort measurements of follicle diameter, so work out the calibration coefficient for the set-up.
15. At the end of the culture, freeze medium and fix or freeze tissue for subsequent analysis, as in step 3.10 above.
16. Follicle-follicle Co-culture.
  1. Culture two follicles together, to investigate interactions between follicles. Culture as above, but place two follicles side-by-side, in contact, in a well.
  2. Place 100 µl droplets of follicle culture medium into each well of a non-tissue culture treated 96-well microtiter flat-well plate, overlaying medium with 100 µl of sterilized silicon oil. Do not transfer follicles into fresh wells as in step 4.13 above, but instead use a fine gel tip to change 50% of the medium every other day.
  3. In order to identify tissue origins within the co-culture, co-culture follicles each from a different genetic source, for example one from the ovary of a wild type mouse, and the other from the ovary of a mouse with ubiquitous expression of GFP. If using GFP or YFP tissue, minimize exposure of tissue to light as much as possible, during culture, and through the fixation/processing steps thereafter.  
NOTE: It is normal for the two follicles to grow together into a single, 'two-follicle' unit (see **Figure 1C**).

## 5. Follicle-Ovary Co-Cultures

1. Dissect ovaries and follicles out as in Sections 3 and 4 above.
2. Place one neonatal ovary on top of a membrane, in a plate prepared as in step 2.4.2 above. Carefully place a single follicle in contact with one pole of the neonatal ovary, using a finely drawn curved glass pipette. Culture in a 37 °C, 5% CO<sub>2</sub> incubator for up to 5 days.
3. In order to distinguish between the tissue origin within the co-culture, use ovaries and follicles from two different sources, for example one from a wild type mouse, and one from a mouse with ubiquitous expression of GFP.
4. Replace 500 µl of medium daily as in step 3.8 above, but using follicle culture medium. During co-culture, the follicle often becomes encapsulated by the ovary (**Figure 1D**).
5. At the end of the culture, freeze medium and fix or freeze tissue for subsequent analysis, as in step 3.10 above.

## 6. Fixation, Immunocytochemistry and Imaging of Cultured Tissue

1. At the end of culture, wash tissue in PBS and transfer to 100  $\mu$ l (follicle) or 1 ml (ovary) of 10% neutral buffered formalin, and fix for 1 hr on ice. Move through 3 washes of 1x PBS at 4 °C.
2. For immunocytochemistry on follicles, transfer between wells of a 96-well microtiter round-well plate using a finely drawn curved glass pipette, with each well containing different washes or treatments.
3. For immunocytochemistry on ovaries (or ovary-follicle co-cultures), embed in 4% agarose gel and section at 50  $\mu$ m using a vibratome. Float sections in the wells of a 24-well plate to apply washes or treatments, removing with a pipette.
4. For imaging: transfer agarose sections to flat glass slides and mount with mounting medium; or transfer follicles (or follicle-follicle complexes) to cavity glass slides and mount with non-hardening mounting medium. Image specimens using a confocal microscope.

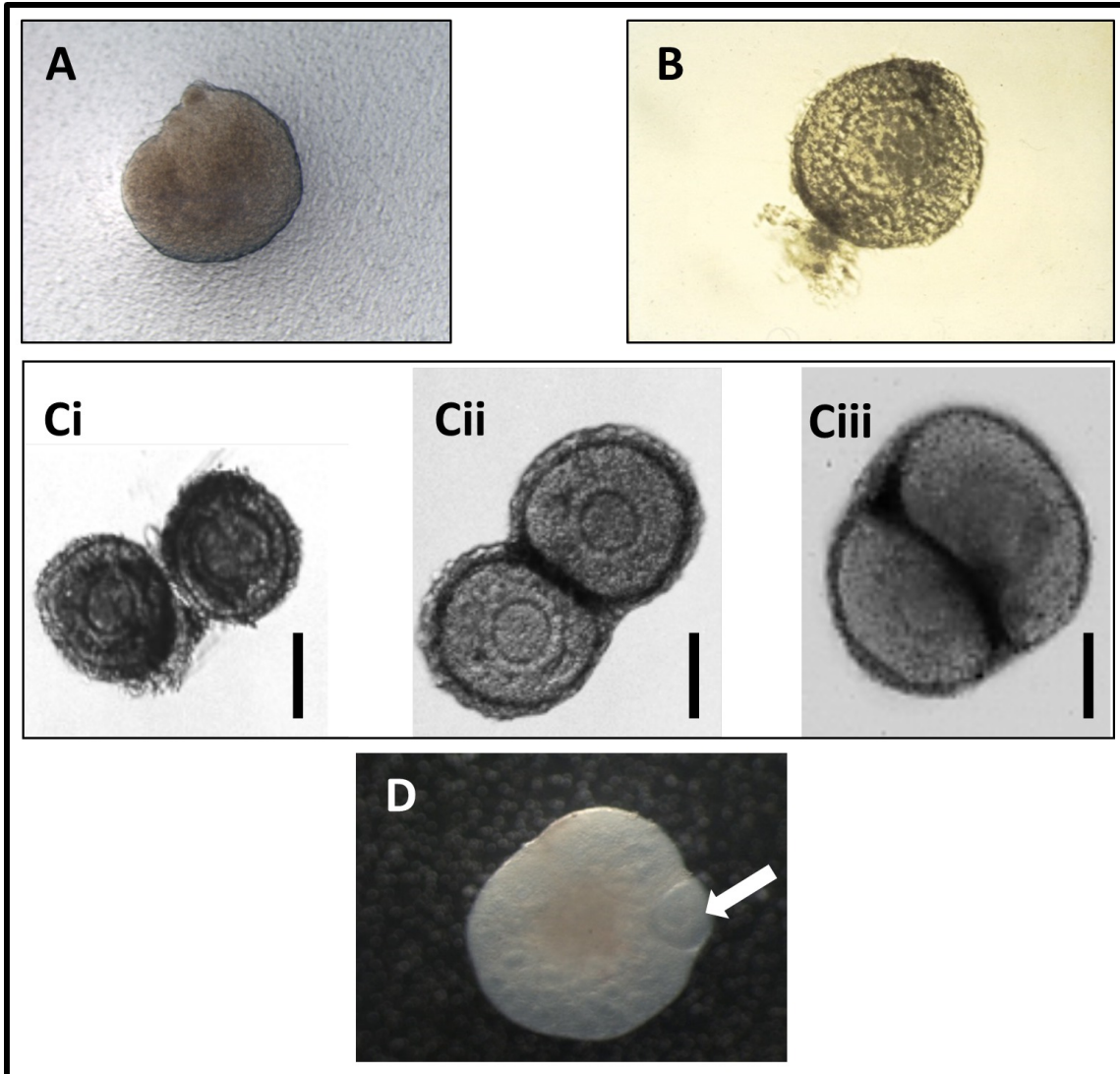
### Representative Results

**Figure 1** shows images of ovaries and follicles at the start of, and during, the culture procedures.

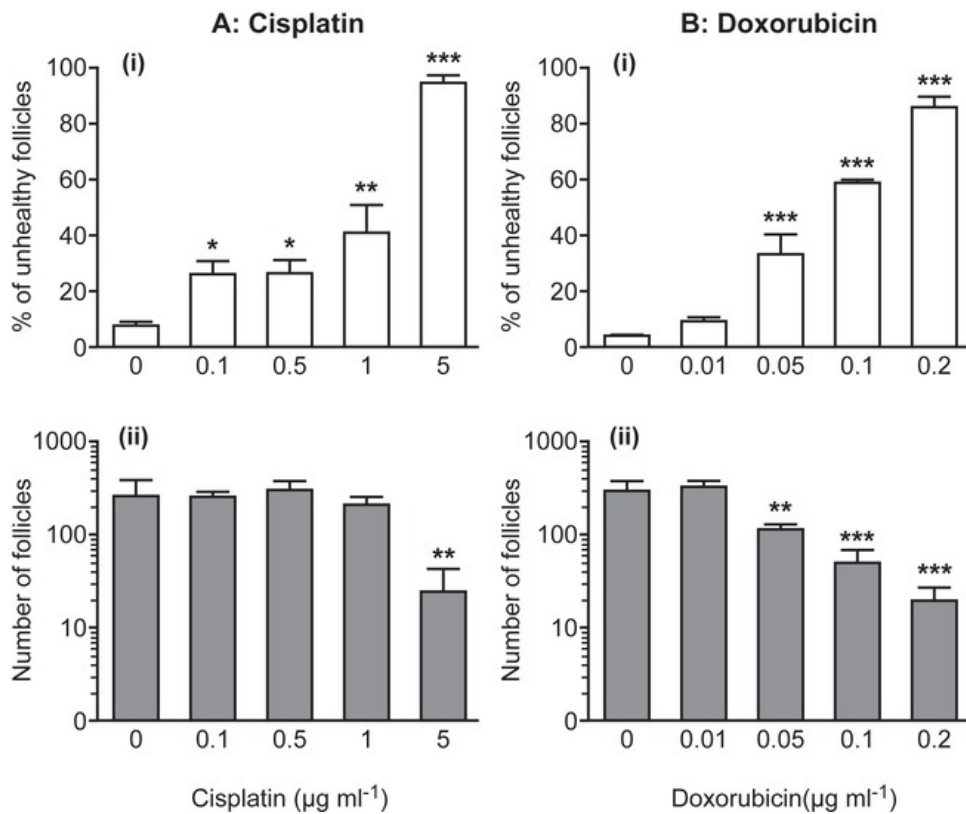
**Figure 2** shows representative results of neonatal ovaries (here, ovaries from newborn pups) exposed to reproductive toxicants during culture. Neonatal ovaries were cultured for 6 days, and exposed to a chemotherapy drug, either cisplatin or doxorubicin, on Day 2 of culture. At the end of the culture, ovaries were fixed, processed for histology, and ovaries then examined to determine the number of healthy and unhealthy ovarian follicles<sup>4</sup>. Alternatively, a more rapid assessment of early growing follicles can be obtained from the culture of ovarian fragments, using ovaries from a mouse expressing an oocyte-specific fluorescent marker<sup>10</sup>.

During the culture of individual follicles, follicle growth can be easily ascertained by daily measurements during the culture period. **Figure 3** shows representative results of the growth of follicles cultured in the presence or absence of the gonadotrophin hormones follicle stimulating hormone (FSH) and luteinizing hormone (LH)<sup>5</sup>.

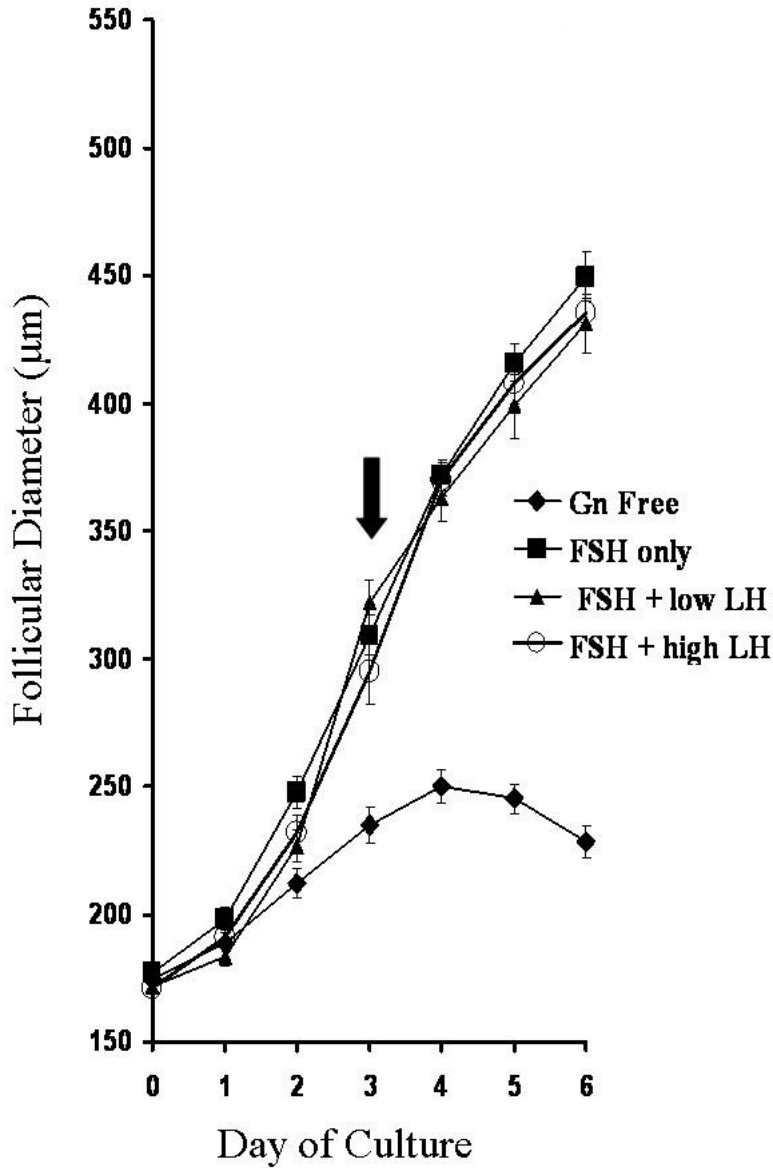
Follicle-follicle or follicle-ovary co-culture can be used to investigate interactions between follicles. Depending on the tissue and processing, images can be obtained using bright-field, fluorescence or confocal microscopes. **Figure 4** shows representative results of images from such co-cultures, examining various cell types involved in follicle-follicle communication, including endothelial and neuronal cells<sup>7</sup>.



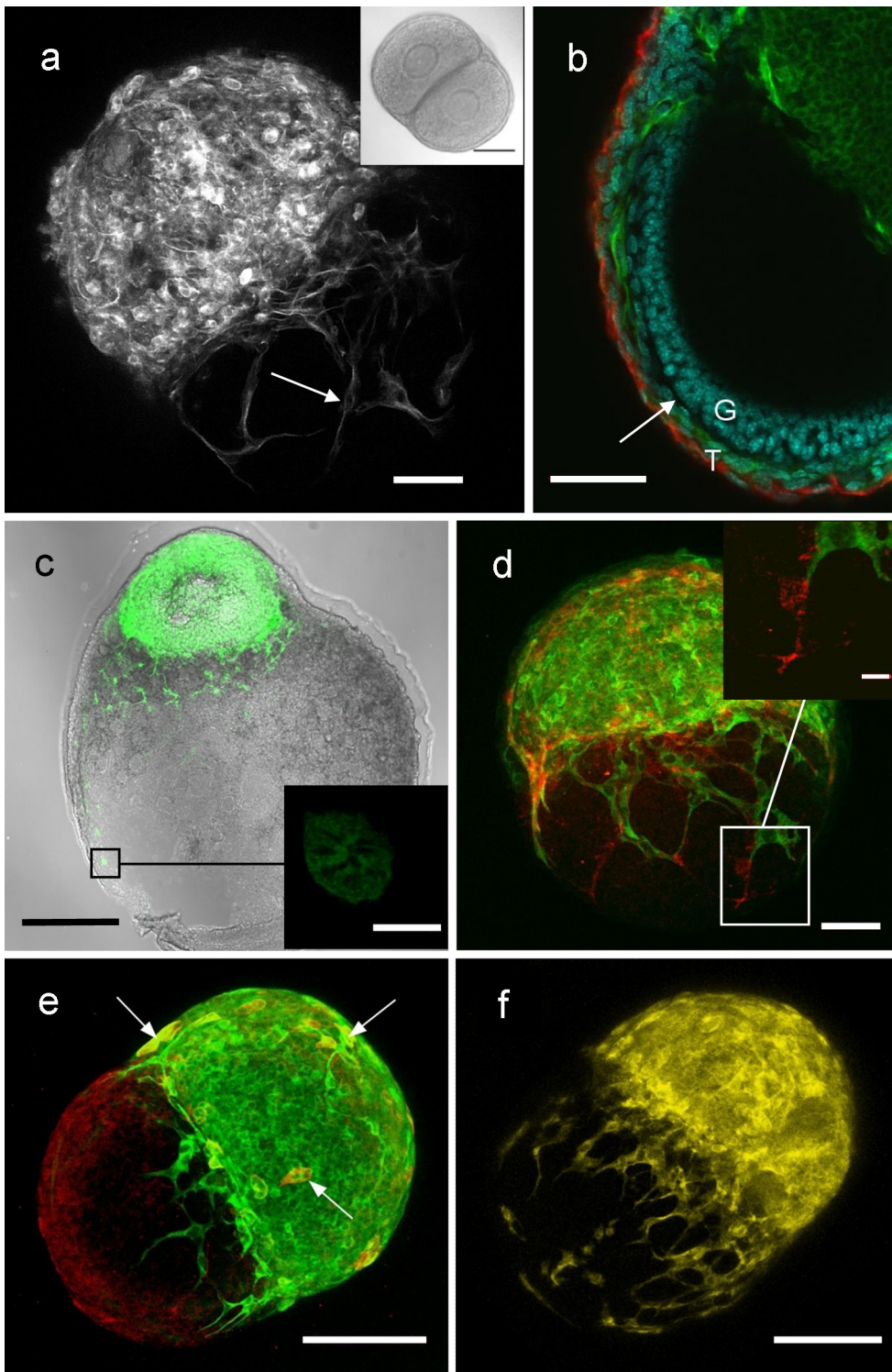
**Figure 1:** (A) Photomicrograph of a neonatal ovary obtained from a mouse on the day of birth, placed on a polycarbonate membrane. (B) Photomicrograph of freshly dissected healthy ovarian follicle, with most of the surrounding stromal tissue dissected away. (C) Photomicrographs of co-culture of two ovarian follicles over the first three days of culture, showing the close contact that develops between the two follicles as the culture proceeds (Ci: first day of culture; Cii: second day of culture; Ciii: third day of culture). Reproduced from Spears *et al.*<sup>11</sup> (D) Photomicrograph of co-cultured preantral follicle and neonatal ovary after two days of culture. Image shows the follicle becoming encapsulated by the ovary. Arrow shows preantral follicle. Image from Dr Federica Lopes. [Please click here to view a larger version of this figure.](#)



**Figure 2: Effect of the chemotherapy drugs cisplatin and doxorubicin on neonatal cultured ovaries.** Cisplatin and doxorubicin both lead to loss of follicle health and a reduction in follicle numbers. (A) Cisplatin; (B) Doxorubicin: (i) Percentage of unhealthy follicles (clear); and (ii) total number of follicles (shaded) in each ovary. Bars denote mean + sem; n = 5 for all groups, stars denote significant differences relative to control (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001). Reproduced from Morgan *et al.*<sup>4</sup>



**Figure 3: Growth rates of follicles exposed to different gonadotrophin environments.** Values are mean  $\pm$  SEM ( $n \geq 16$  for each group). Arrow indicates the start of antral formation in all gonadotrophin groups. Reproduced from Murray *et al.*<sup>5</sup>



**Figure 4: Images from follicle-follicle and ovary-follicle co-cultures, showing follicle-follicle interactions.** (A) Confocal micrograph of a green fluorescent protein (GFP) follicle and a wild-type (WT) follicle after co-culture (GFP expression shown here in white), with processes from the GFP follicle shown extending over the WT follicle. (B) Cross-section through a GFP/WT follicle complex following co-culture, showing that GFP processes (green) from the adjoining follicle lie adjacent to the basal lamina of the WT follicle. (C) Neonatal WT ovary following culture with a pre-antral GFP-expressing follicle, showing that GFP cells and cellular processes (green) have migrated through the ovarian interstitium of the neonatal ovary. (D) GFP/WT follicle complex containing endothelial cells, shown by expression of endothelial cell marker CD31 (red). (E) GFP/WT follicle complex containing neuronal cells, shown by expression of neuronal marker beta tubulin III (see here as red, or as yellow if double-labelled with GFP, green). (F) YFP/WT follicle complex, where YFP refers to follicle from a Thy1-YFP mouse that has occasional expression of yellow fluorescent protein (YFP: yellow) in a subset of neuronal cells<sup>9</sup>, showing that neurons extend from the YFP follicle. Bars 50 μm (A, B, D), 100 μm (C, E, F), 100 μm (inset in A), 10 μm (inset in C), 15 μm (inset in D). Reproduced from Campbell *et al.*<sup>7</sup> [Please click here to view a larger version of this figure.](#)



## Discussion

We describe here various culture systems that can be utilized to support the development of mouse ovarian follicles *in vitro*, using whole neonatal ovaries containing only the earliest follicle stages, individual preantral ovarian follicles and also co-cultures of the two tissues.

Culture of neonatal mouse ovaries supports early ovarian development, particularly follicle growth initiation up to the secondary follicle stage. A range of ages of neonatal mice can be used for these cultures, depending on the developmental stages of interest. If ovaries are obtained from newborn mice, follicle formation will be underway but not yet complete: culture will at least partially support continued follicle formation followed by follicle growth. Alternatively, use of ovaries from mice around four-to-five days of age (by which time follicle formation is already complete) results in culture of a greater number of primordial and growing follicles<sup>12</sup>. Beneficial aspects of the specific technique described here include use of floating polycarbonate membranes, which allow greater oxygenation of the tissue, and culture in a very basic medium consisting of only  $\alpha$ MEM and BSA, avoiding the use of undefined additives such as serum. Culture of whole ovaries does not seem to support development beyond the secondary follicle stage, with subsequent development requiring a change in technique, such as the dissection of follicle-granulosa cell complexes from the cultured neonatal ovary<sup>1</sup>.

Later stages of follicle development can be developed *in vitro* by dissecting out individual, intact late pre-antral follicles, which can be grown to the preovulatory stage in culture whilst maintaining their three-dimensional structure. The use of intact follicles for this culture technique maintains the relationship between the different follicular components as occurs *in vivo*. This culture system can be used to obtain oocytes that can support fertilisation and subsequent embryo development.

Fertilizable oocytes can also be obtained from mouse neonatal ovaries using an initial culture protocol much as described here, followed by a second stage during which oocyte-granulosa cell complexes are grown *in vitro*<sup>1</sup>. Other systems used fairly frequently today include culture of follicles or ovarian tissue that has been encapsulated in a material such as alginate hydrogel, to provide support (see, for example, Tagler *et al.*<sup>13</sup>). Much of the focus of method development now is to improve culture techniques for the ovaries and follicles of larger mammals, with the long-term aim of obtaining fertilisable oocytes from primordial follicles from a range of species, including humans.

At any one time, mammalian ovaries contain follicles at a range of developmental stages, with interactions between follicles affecting their regulation. This aspect of ovarian function is poorly understood and difficult to examine *in vivo*. The final method described here uses co-culture systems to support development of different stages of follicles *in vitro*. If required, one or both tissues can be pre-treated *in vivo* or *in vitro* prior to co-culture. Co-culture systems such as this provide an ideal way in which to examine follicle-follicle interactions, for instance how growing, antral follicles affect the primordial follicle pool, aspects of ovarian biology that have proven difficult to examine until now.

Whole ovary culture techniques are fairly straightforward, although careful dissection is required to avoid accidental tissue damage. Dissection of individual follicles is a specialised technique, requiring repeated practice before follicles at the right stage can be dissected out from the ovary intact and undamaged. It is critical to dissect out individual follicles carefully, or damage sustained during the dissection protocol can result in follicle death during the subsequent culture period. Where follicles are placed directly into the well of microtiter plates, it is important to use only non-tissue culture treated plastics, to minimise plating down of thecal cells on the plastics: if tissue culture treated plasticware is used, the follicles will attach to the base of the well and rupture as they grow. For all co-culture work, tissues must be placed directly in contact with each other.

The medium detailed above for use in the follicle culture technique includes the addition of mouse serum. It is possible to replace the mouse serum with fetal bovine serum, but only occasional batches of such sera will fully support follicle development to the preovulatory stage, with batch-testing required to identify suitable sources. Batch-testing of FSH is also advisable, as the International Units by which FSH is assessed correlate only crudely to follicle growth *in vitro*. If follicle rupture routinely occurs during the culture period, consider replacing stock ascorbic acid with a fresh batch.

The techniques do not require particularly specialised equipment other than dissecting microscopes and tissue culture incubators, although use of a laminar flow hood and good sterile technique allow the ovarian follicles to be cultured in the absence of antibiotics, as in the methods described here. This can be helpful, to avoid any potential detrimental effect of antibiotics on the oocytes, particularly if they are to be fertilized following culture. Where it is not possible to work in a sterile environment, it is advisable to add antibiotics to the dissection and culture media.

## Disclosures

The authors have nothing to disclose.

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