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Ovarian Tissue Culture to Visualize Phenomena in mouse ovary

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Author Comments:	<p>In this article, we show the method of ovarian tissue culture. This ovarian tissue culture enables to observe the dynamics in mouse ovary, follicle development, ovulation and follicle atresia. Mammalian ovary contains numerous follicles and ovulates periodically. However, it is not clear how mammalian ovary controls the follicle development to sustain the periodic ovulation. To study the regulatory mechanism of follicle development in mouse ovary, we developed the method of ovarian tissue culture and observation of the dynamics in cultured ovary. We confirmed that cyclic follicle development and ovulation are reproduced under culture condition by time-lapse imaging. Under the culture condition, we can trace the process from primordial follicle to ovulation, so this method is useful to clarify the mechanism of follicle development and other phenomena in mouse ovary.</p> <p>In the regulatory mechanism of follicle development, the activation of dormant primordial follicle is one of important research subjects. To clear the mechanism of activation of primordial follicles in the ovary, it needs to visualize the process of primordial to primary follicle transition at real time. However, follicle stages are classified by morphological character of granulosa cells, so it is difficult to observe the activation of primordial follicle in real time. In this article we present a new method to visualize primordial to primary follicle transition, using oog1pro3.9 transgenic mouse. This method is useful for the research about activation of primordial follicle. These methods must contribute the research about ovarian phenomena, so we submit this article to Journal of Visualized Experiments</p>
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TITLE:

Ovarian Tissue Culture to Visualize Phenomena in Mouse Ovary

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KEYWORDS:

Ovary, tissue culture, follicle development, primordial follicle, ovulation, follicle atresia, oogenesis 1, time-lapse imaging

SHORT ABSTRACT:

Ovarian tissue cultures can be used as models of follicle development, ovulation, and follicle atresia and indicate regulatory mechanisms of dynamic ovarian processes.

LONG ABSTRACT:

Mammalian females periodically ovulate an almost constant number of oocytes during each estrus cycle. To sustain such regularity and periodicity, regulation occurs at the hypothalamic-pituitary-gonadal axis level and on developing follicles in the ovary. Despite active studies, follicle development mechanisms are not clear because of the several steps involved from the dormant primordial follicle activation to ovulation, and because of the regulation complexity that differs at each follicular stage. To investigate the mechanisms of follicle development, and the dynamics of follicles throughout the estrus cycle, we developed a mouse ovarian tissue culture model that can be used to observe follicle development using a microscope. Systematic follicle development, periodical ovulation, and follicle atresia can all be reproduced in the cultured ovary model, and the culture conditions can be experimentally modulated. Here, we demonstrate the usefulness of this method in the study of the regulatory mechanisms of follicle development and other ovarian phenomena.

INTRODUCTION:

Female mouse ovaries contain several thousand follicles¹, and periodic ovulation matures approximately ten oocytes at each estrus cycle. Follicles are classified into several developmental

stages: primordial, primary, secondary, antral, and Graafian follicles, depending on the form of the granulosa cellular layer surrounding each oocyte. Most primordial follicles are dormant, and some of them are activated and grow into primary follicles at each estrus cycle². After the secondary follicular stage, follicle development is mainly regulated by gonadotropins, follicular stimulating hormone (FSH), and luteinizing hormone (LH). However, primordial and primary follicle development is independent of gonadotropin, and the regulatory mechanisms that govern these stages remain poorly understood³⁻⁵. In addition to growth factors and hormones, the primordial and primary follicle is regulated by the interactions among follicles^{6,7}. Therefore, we performed analyses of follicle dynamics in mouse ovary tissues, and investigated the associated regulatory mechanisms using ovarian tissue cultures⁸⁻¹⁰.

Herein, we introduce two ovarian tissue culture model methods. The first is used to analyze follicle development by measurement of follicular areas, and the second is used to study the regulatory mechanism during early follicle development from primordial to secondary follicle stage with transgenic mice. For follicle development analysis, we mainly used ovaries of 4-week old female mice because they allow for easy visualization of follicles. To induce periodical ovulation and model *in vivo* follicle development, we reproduced LH surge and observed ovulation, follicle atresia, and secretion of estradiol under tissue culture conditions. Images of the cultured ovaries were captured, and the follicle development processes were analyzed by tracing changes in the follicular area. However, in bright field microscopy analyses, the distinction between primordial and early primary follicles was unclear. Thus, we developed a method to detect small follicles, and distinguish between the primordial, primary, and secondary follicle in cultured ovarian tissues using *Oogenesis1 (Oog1) pro3.9* and *R26-H2B-mCherry* transgenic mice ovaries at days 0 and 4 after birth¹¹. *Oog1* expression is detectable in oocytes after entry into meiosis, and gradually increases with follicle development, allowing observation of the transition from primordial to primary follicles using time-lapse images of cultured ovary tissue^{11,12}. Although morphological methods have been used to study factors that activate dormant primordial follicles¹³⁻¹⁶, physiological follicle development in ovaries is difficult to observe, and the effects of various factors remain uncharacterized. The present culture methods were designed to address this paucity in real time analyses of target factors.

In the present study, we tracked follicular development using a time-lapse imaging method and characterized the process of follicle development. Our novel methods offer an unprecedented tool for investigating the physiology of ovaries.

PROTOCOL:

Mice were housed in an environmentally controlled room at 23 ±1 °C with a 12-h light/12-h dark cycle. Animal care protocols and experiments were conducted in accordance with the Guidelines for Animal Experimentation at Aichi Medical University and were approved by the incumbent Animal Care and Use Committee.

1. Preparation of Culture Medium and Dishes

1.1. To prepare basic culture medium, add fetal bovine serum (FBS, 5% v/v), FSH (100 mIU/mL), LH (10 mU/mL), and penicillin-streptomycin (penicillin, 100 U/mL; streptomycin, 100 mg/mL) to minimum essential medium alpha (MEM-alpha) and mix in 50 mL tubes.

Note: Total volumes of required culture media vary between experiments, but 1 mL of culture medium is generally sufficient for 3.5-cm glass-bottom culture dishes.

1.2. Pour 1-mL aliquots of mixed culture medium into 3.5-cm glass-bottom culture dishes and set place 30-mm cell culture inserts into dishes. Pre-warm media and dishes in an incubator (5% CO₂ and 37 °C).

2. Preparation of Ovarian Tissue

2.1. Pre-warm phosphate buffered saline (PBS) (–) and MEM-alpha containing 5% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in an incubator (5% CO₂ and 37 °C).

Note: About 3-mL aliquots of PBS (–) per ovary are sufficient for use during ovary dissections, and 1-mL aliquots of MEM-alpha are sufficient for transient storage of single ovaries in 3.5-cm dishes.

2.2 Excise ovaries from 4-week-old female ICR (named after Institute of Cancer Research) mice and trim the tissues surrounding the ovary using scissors and tweezers under a stereoscopic microscope. Reserve the removed ovaries in culture medium (see step 2.1) until use.

2.2.1. Place single ovaries onto filter paper moistened with pre-warmed PBS (–) (see step 2.1) and slice into 4 pieces using a microtome blade under a stereoscopic microscope.

Note: Ovaries of 4-week-old ICR mice are about 2 mm in diameter. The number of pieces depends on the ovary size; however, about 500 µm-thick pieces allow for proper follicle observations (in pieces thicker than 500 mm, tissue transparency is decreased; in pieces thinner than 500 mm, many antral follicles are broken and lost).

2.2.2. After dissection, place the sliced ovary specimens into pre-warmed culture medium in 3.5-cm dishes (see step 2.1).

3. Ovarian Tissue Culture

3.1. Drop approximately 0.5 µL of culture medium per sliced ovarian tissue on the cell culture insert where the ovarian tissue will be set using a micropipette.

3.2 Place each sliced specimen into a drop of culture medium on the cell culture inserts using tweezers.

3.3 Culture the ovary tissues in 5% CO₂ at 37 °C.

132 3.4. Replace the culture medium with fresh pre-warmed medium every 2 days.

133
134 Note: The schedule for medium changes for the culture of ovary sections from 4-week-old ICR
135 mice is presented in **Figure 1**.

136
137 3.4.3.1. To reproduce the LH surge, treat cultured 4-week-old ICR mice ovaries with the medium
138 containing 100 mU/mL FSH and LH for 12 h every 4 days (**Figure 1**).

139 140 4. Microscope Images of Cultured Ovaries

141
142 4.1 Start imaging the cultured ovaries after day 1 when the tissues have adhered onto the cell
143 culture inserts, and perform confocal or inverted microscope analyses at 24-h intervals to allow
144 sufficient follicle growth between time points.

145
146 Note: Confocal microscopy is superior to inverted microscopy for observing follicles in whole
147 cultured ovaries.

148 149 5. Time-lapse Imaging of Cultured Ovaries

150
151 5.1. Optimize the time-lapse imaging conditions, including laser intensities and exposure times,
152 for the imaging system (**Table 1**).

153
154 5.1.1 Select paired ovary specimens from single mice for use as treatment and control groups.
155 Vary laser intensities and exposure time to achieve the best images (**Table 1**).

156
157 5.1.2. Compare follicle growth under each culture condition by measuring follicle areas in images
158 of control samples at 24-h intervals and in time-lapse imaging samples (see step 6). Concurrently,
159 count the number of ovulated oocytes in each ovary set and choose the optimal time-lapse
160 imaging conditions.

161
162 5.2. Capture images at 30-min intervals using the time-lapse imaging system under the
163 determined conditions (**Table 1**).

164 165 6. Follicle Growth Analysis

166
167 6.1. To analyze the follicle development, measure the follicle areas in the captured images using
168 ImageJ software (<http://rsbweb.nih.gov/ij/>).

169
170 6.1.1 Initially, set the scale of the image by clicking **Set Scale** under **Analyze** in the tool bar. Enter
171 the side lengths of the captured image and the corresponding numbers of pixels in the blank fields
172 of **Known distance** and **Distance in pixels**, respectively.

173
174 6.1.2. Click **Free hand** in the tool bar and outline the follicles in the captured bright field images.

175

176 **6.1.3. Click *Measure* under *Analyze* in the tool bar.**

177
178 Note: If other measurement data are desired, click **Set measurement** under **Analyze** in the tool
179 bar, and check the appropriate boxes in the list.
180

181 **7. Analysis of Follicle Development Using Transgenic Mice**

182
183 7.1 Collect ovaries from postnatal days 0 and 4 (P0 and P4) female transgenic mice containing the
184 transgenes *Oog1pro3.9* and *R26-H2B-mCherr*, and culture on inserts as described in steps 1.1–
185 3.2, except do not slice the P0 and P4 ovaries.
186

187 7.2 Replace the culture medium with fresh, pre-warmed medium in 3.5-cm dishes in an incubator
188 containing 5% CO₂ at 37 °C every 2 days.
189

190 Note: The concentration of LH in the culture medium of P0 and P4 ovaries can remain constant
191 because LH surges do not occur in P0 and P4 mice.
192

193 7.3 Set the microscope to visualize only AcGFP1-positive primary follicles in cultured P4 ovaries
194 (**Table 1**).
195

196 Note: Only primordial and primary follicles are present in P4 female mouse ovaries.
197

198 7.4 Capture images of cultured P0 *Oog1pro3.9/R26-H2B-mCherry* transgenic mice ovaries at 30-
199 min intervals using the settings used for P4 ovaries (see step 5.2).
200

201 7.5 Trace and analyze follicle development using AcGFP1 and H2B-mCherry signals.
202

203 **REPRESENTATIVE RESULTS:**

204 **Figure 1** shows the protocol for changes in media during ovarian tissue culture. Following this
205 program, 4-week-old ICR mice ovaries were cultured and imaged at 24-h intervals using confocal
206 microscopy (**Figure 2**). During culture of ovary tissues for 3 weeks, most antral and secondary
207 follicles were degenerated by follicle atresia and some were ovulated (**Figure 2D** and **Table 2**). In
208 analysis of follicle areas using ImageJ (**Figure 3**), some follicles developed in groups during each
209 LH surge cycle (**Figure 3B** and **Supplementary Movie 1**). Moreover, ovulation occurred almost
210 simultaneously in separate cultured ovaries from right and left ovaries of single mice. Whereas,
211 the timing of ovulation and numbers of ovulated oocytes (**Table 2**) differed between mouse
212 ovaries (data not shown), ovulation from cultured ovaries predominantly occurred within 48 h of
213 LH surges (**Figure 3**, and **Supplementary Movie 1**). However, not all ovulated oocytes released
214 first polar bodies after ovulation (**Figure 2E** and **Table 2**), and although ovulated oocytes could be
215 fertilized, development ceased at the 4-cell stage (data not shown). To elucidate mechanisms by
216 which dormant primordial follicles are activated in mouse ovaries, we detected primordial follicle
217 activation in cultured tissues from transgenic mice carrying *Oog1pro3.9* and *R26-H2B-mCherry*
218 transgenes (**Figure 4**, **Figure 5**, and **Supplementary Movie 2**). Oocytes express very low levels of
219 the *Oog1* gene from the primordial follicle stage, and time-lapse imaging distinguished low

AcGFP1-expressing primordial follicles from the high AcGFP1-expressing primary follicles. Primordial and primary follicles are present simultaneously in P4 mouse ovaries, whereas only primordial follicles are present in P0 ovaries (**Figure 4A, B**). Thus, we optimized time-lapse imaging conditions to detect only primary follicles in cultured P4 ovaries (**Figure 4C**). Subsequently, we captured images of cultured P0 ovaries for 10 days in the same conditions (**Figure 4D–G**). AcGFP1 can be used as an index of primary follicles in these transgenic mice and AcGFP1-positive primary follicles were detectable in cultured P0 ovaries after 30–40-h culture (**Figure 4, Figure 5A–F**, and **Supplementary Movie 2**). Primordial and primary follicles in cultured ovaries were also distinguished by mCherry-positive nuclei of granulosa cells (**Figure 5B, E**). Specifically, mCherry signals indicate forms of follicles and allow observation of follicle stages in cultured ovaries (**Figure 5B, E**, and **H**). Therefore, this culture system using ovaries from *Oog1pro3.9/R26-H2B-mCherry* mice revealed the process of early follicle development from primordial to secondary follicle stages. These methods will facilitate studies of the regulatory mechanisms of gonadotropin-independent follicle development.

FIGURES AND TABLE LEGENDS:

Figure 1: Time course of medium changes. Medium A contains 5% FBS, 100-mU FSH, 10-mU LH, 100-U/mL penicillin, and 100-mg/mL streptomycin in MEM-alpha. Medium B contains 5% FBS, 100-mU FSH, 100-mU LH, 100-U/mL penicillin, and 100-mg/mL streptomycin in MEM-alpha. Medium B was used to produce LH surges every 4.

Figure 2: Images of cultured ovarian tissues. Images were captured at 24-h intervals using confocal microscopy. (A) Culture day 1; (B) Culture day 5; (C) Culture day 10; (D) Culture day 13; (E) Magnified image of the region indicated by the square in (D); oocytes in D were ovulated from the follicles indicated by arrows in B, C, and D. (F) Oocyte releasing a polar body after ovulation; scale bar, 200 μ m.

Figure 3: Measurements of follicle areas in cultured ovaries. (A) Image of cultured ovary; dotted lines represent the area measured by ImageJ. (B) Follicular areas in cultured ovary after 3 weeks; gray lines represent the culture period from the addition of 100-mM LH (LH surge). Lines show the development stages in each follicle in the cultured ovary; scale bar: 200 μ m.

Figure 4: Hematoxylin and Eosin (H&E) staining images of P0 and P4 ovaries and time-lapse imaging. (A) H&E staining of P0 ovary; (B) H&E staining of P4 ovary; (C) Image of a P4 ovary from an *Oog1pro3.6* transgenic mouse after 10-h culture. The arrow shows an AcGFP1-positive primary follicle. (D–G) Images of P0 ovaries from transgenic mice expressing *Oog1pro3.9* and *R26-H2B-mCherry*; green and red signals in D and F represent AcGFP1 and mCherry, respectively. White signals in E and G represent AcGFP1 in D and F, respectively. D and E are images of cultured ovaries after 0.5-h culture. F and G show ovaries after 180-h culture; scale bar: 100 μ m.

Figure 5: Images of primordial, primary, and secondary follicles in cultured ovaries from *oog1pro3.9/R26-H2B-mCherry* mice. (A–C) Images of primordial follicles in cultured P0 ovaries; (D–F) Images of primary follicles in P0 cultured ovary; (G–H) Images of secondary follicles in

cultured 4-week-old mouse ovaries. A, D, and G are merged images of B and C, E and F, and H and I, respectively. Green, AcGFP1; red, mCherry; scale bar: 100 μ m.

Table 1. Time-lapse imaging conditions. Time-lapse imaging condition for confocal and bright field microscopes.

Table 2. Numbers of ovulated oocytes in cultured ovaries. Numbers of ovulated oocytes from twelve cultured ovaries; MII indicates the numbers of ovulated oocytes that released first polar bodies after ovulation.

Supplementary Movie 1: Time-lapse movie of a cultured ovary. Ovaries were collected from 4-week-old mice and were sliced and cultured for 3 weeks. The movie spans a culture period of 0–200 h at 10 frames/s.

Supplementary Movie 2: Time-lapse movie of a cultured P0 ovary from a transgenic mouse. Green, AcGFP1; red, mCherry. The movie spans a culture period of 0–180 h at 10 frames/s.

Supplementary Movie 3: Time-lapse movie of a cultured ovary from a 4-week-old mouse. Green, AcGFP1; red; mCherry; the movie spans a culture period of 9–13 culture days at 10 frames/s. The arrows in the first image indicate follicles in which atresia occurred during culture.

DISCUSSION:

In this study, we developed two new methods for studying follicle development in mouse ovaries. The first method involves culture of sliced ovarian adult mice tissues followed by analyses of follicle development, and the second involves the use of time-lapse imaging to visualize early follicle development during the gonadotropin-independent stage. Previously, we used the present ovary tissue culture method to assess the effect of leukemia inhibitory factor and progesterone on follicle development^{8,9}, and showed that their effects vary with concentration and follicle stage. In the present study, sliced ovarian tissues exhibited follicle dynamics, warranting further analyses of ovarian follicle development mechanism using this model.

Ovaries from mice of more than 5 weeks of age contain corpus lutea that hinders microscopic observations. Hence, 4-week-old mouse ovaries are preferred for observations of follicle development and ovulation, and subsequent late primary, secondary, and antral follicles are more easily distinguished using bright field microscopy. Our methods offer comparisons of the effects of growth factors and drugs in culture media, with discernable changes in follicle development between differentially treated ovaries (**Figure 3**).

Transgenic mice expressing Cre or fluorescein proteins in oocytes have been described in previous studies^{18–20}. However, follicular stage classification depends on histological characteristics including granulosa cell shapes, numbers of granulosa cell layers, and whether theca cells are formed. Thus, distinctions between primordial and primary follicles may be limited from observations of oocytes alone. Herein, we used transgenic mice containing the *Oog1pro3.9* and *R26-H2B-mCherry* transgenes^{11,17} and visualize early follicle development from primordial to

secondary follicle stages in cultured ovaries. Oog1 expression becomes detectable in oocytes during the primordial follicle stage, and is markedly increased in primary follicles. Hence, the AcGFP1 signal intensities in oocytes are associated with follicle stage (**Figure 5**) and were used to observe primordial-primary follicle transitions (**Figure 4, Supplementary Movie 2**). Cell nuclei are stained red in *R26-H2B-mCherry* transgenic mice (**Figure 4, Supplementary Movie 2, and Supplementary Movie 3**), allowing observations of follicle stages according to numbers of granulosa cells (**Figure 5**). Thus, collectively the present methods allow analyses of follicle development from primordial through to ovulation using *Oog1pro3.9/R26-H2B-mCherry* transgenic mice. Furthermore, because apoptotic cell chromatin is condensed, mCherry signals of atretic follicles are stronger than those of normal follicles (**Supplementary Movie 3**).

Among methodological subtleties, correct thicknesses of sliced tissues are required for successful culturing, with increased cell death in ovary slices that are too thick, and losses of large antral follicles in ovary slices that are too thin. The follicle growth speed is slow; hence, it is easy to trace and analyze the process of each follicle via 24-h interval observation. The present confocal microscope setting, including laser intensity, interval time, and digital gain, are dependent on the microscope mode, but are critical to consider when obtaining time-lapse images. In particular, strong laser intensities and long exposure times are required to capture clear images, but can affect cultured cells. Hence, moderation of these settings to avoid phototoxicity is critical. Sliced ovarian tissues of 4-week-old mice can be cultured for approximately 4 weeks, whereas primordial and primary follicles remain present subsequently, they no longer grow. In contrast, P0 and P4 ovaries can be cultured for approximately 10 days, during which primordial and primary follicles develop into primary or secondary follicles, respectively. However, follicles in cultured P0 and P4 ovaries do not develop into antral follicles. Therefore, the excision of ovaries is required at various stages for studies of the regulatory mechanisms associated with whole ovary follicle development.

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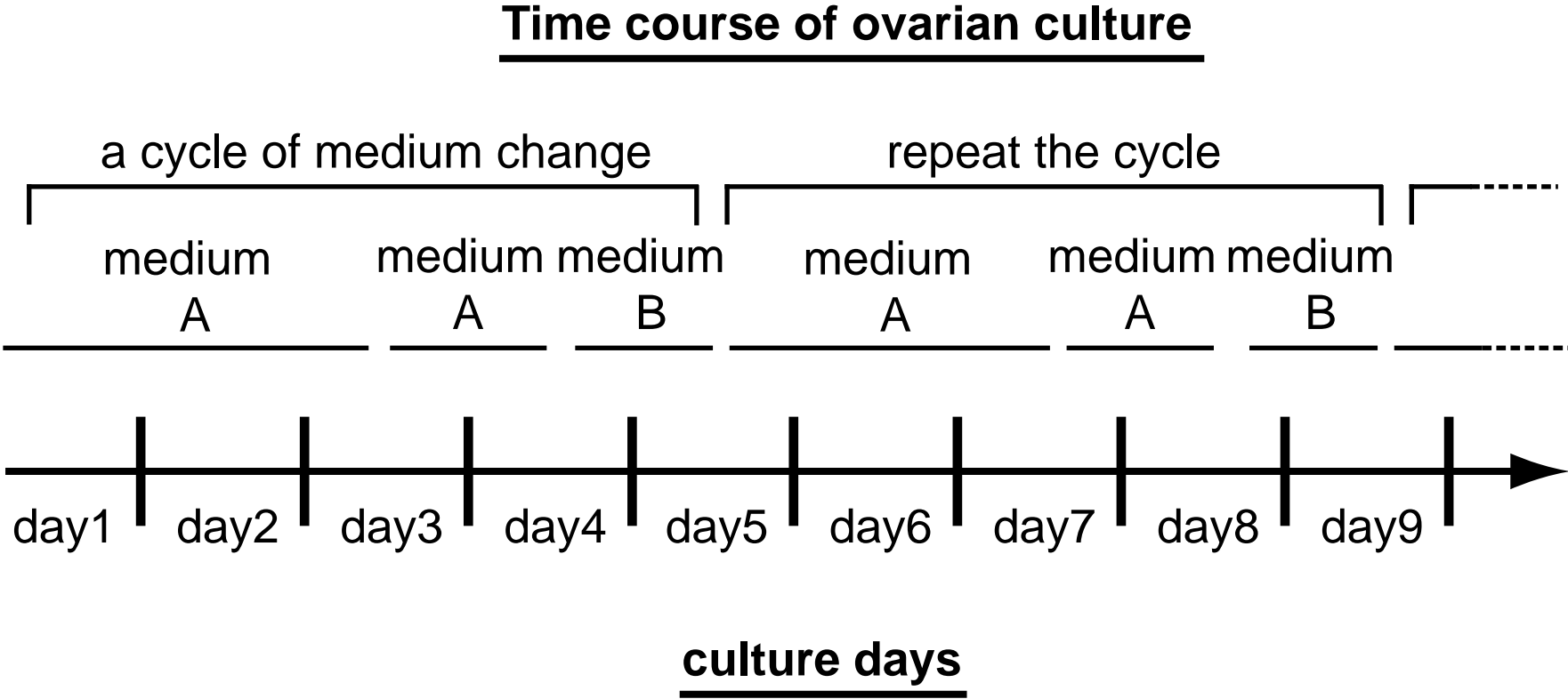
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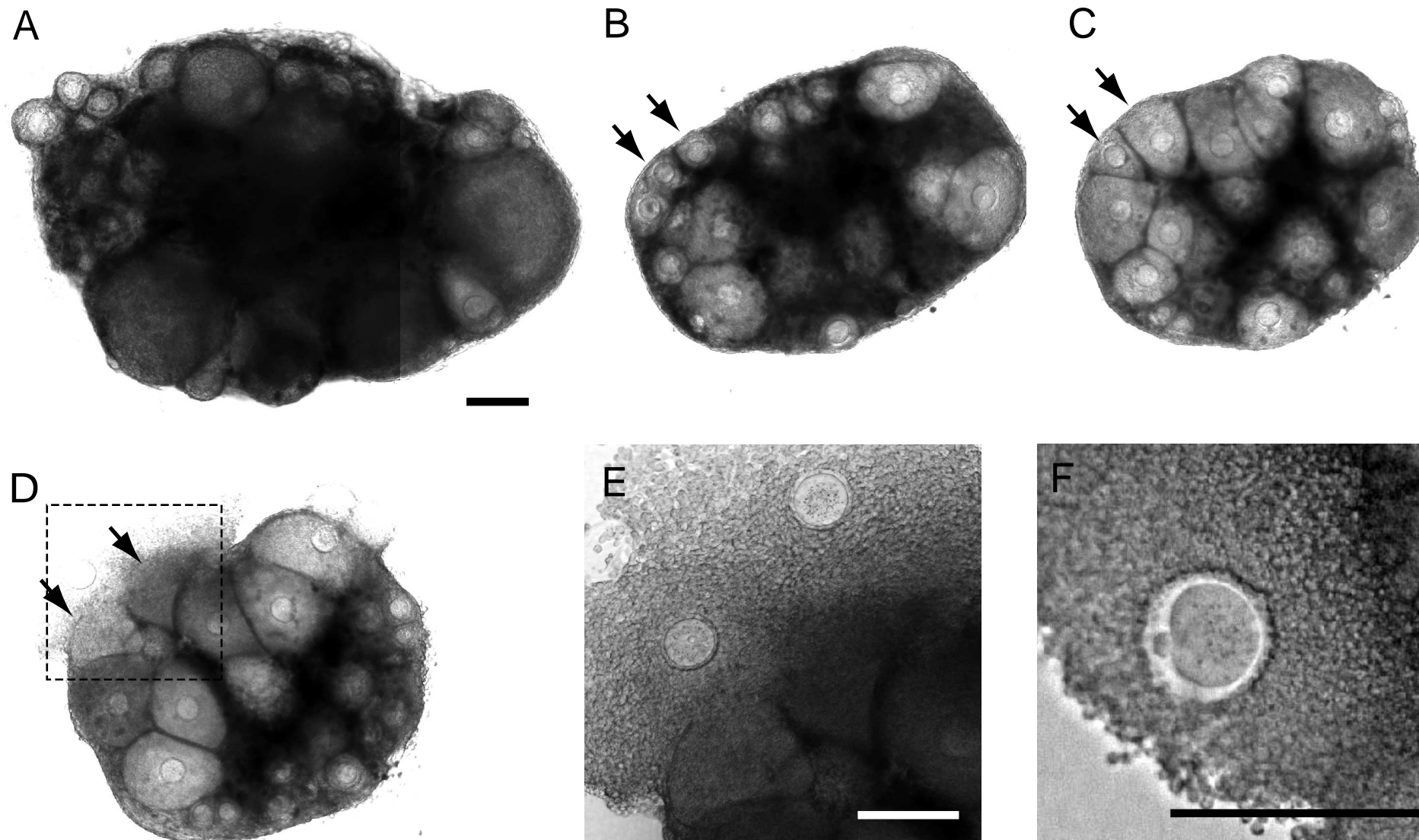
The authors have nothing to disclose.

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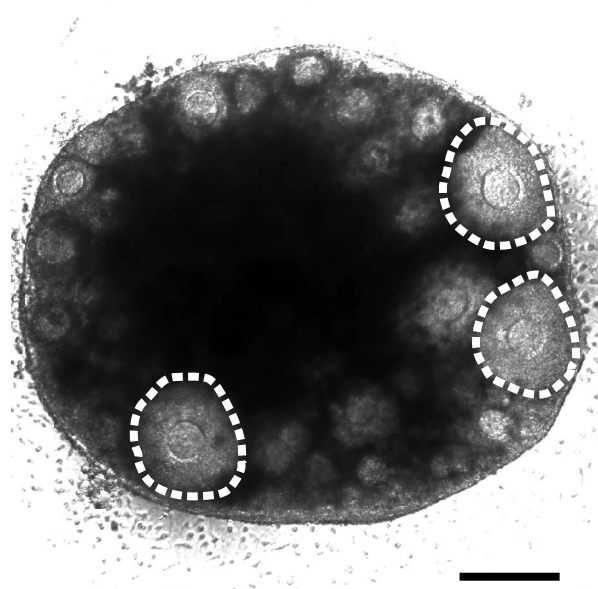
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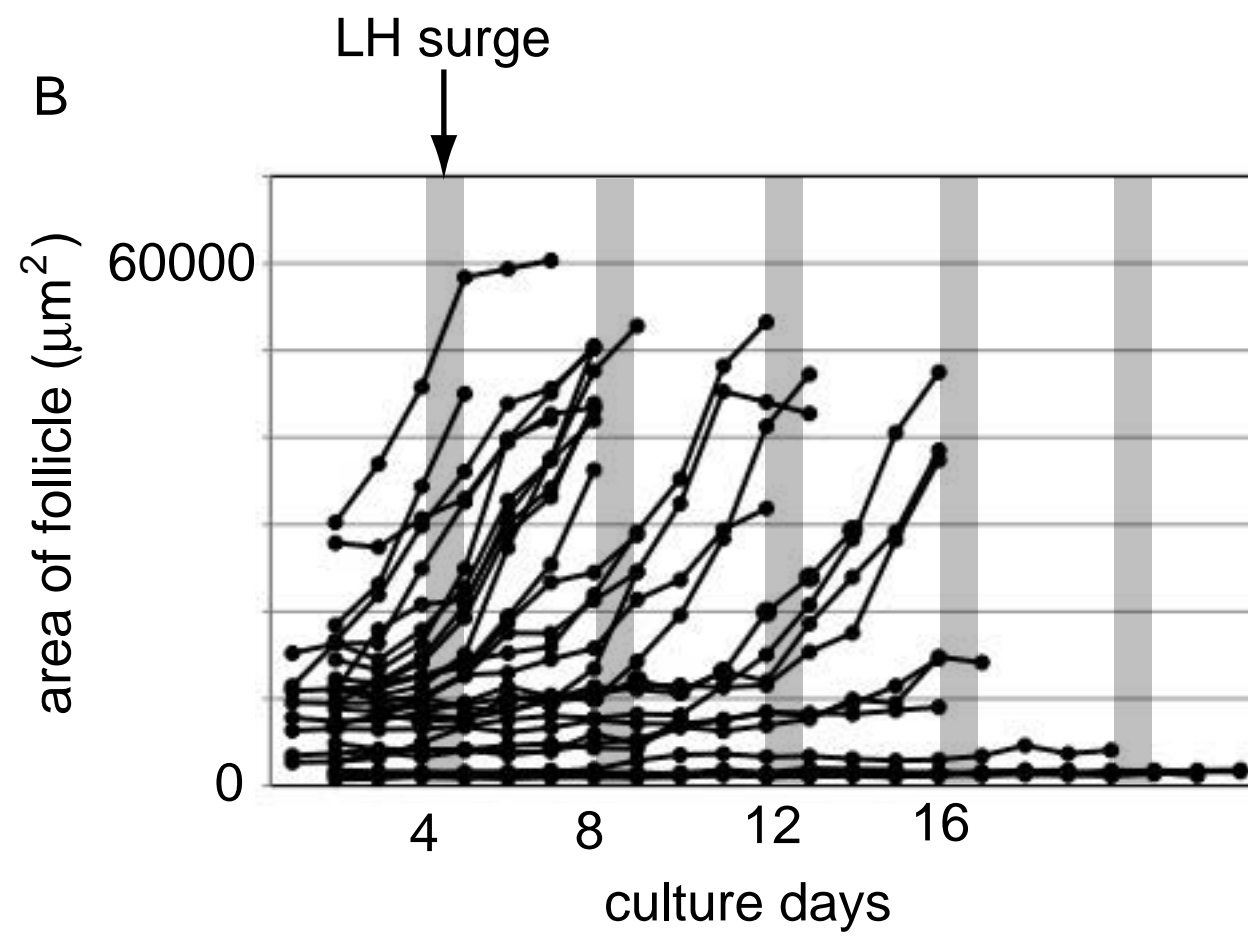


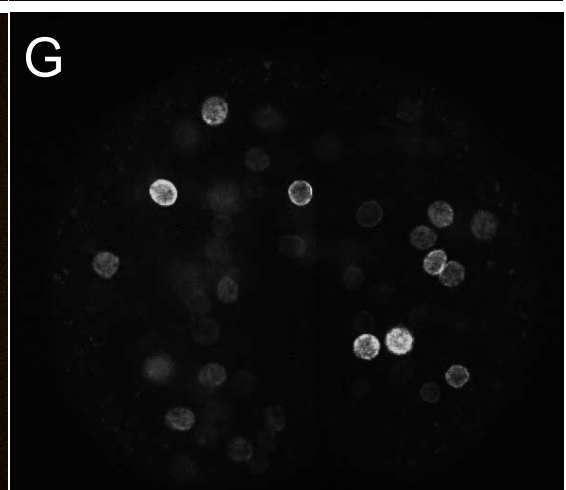
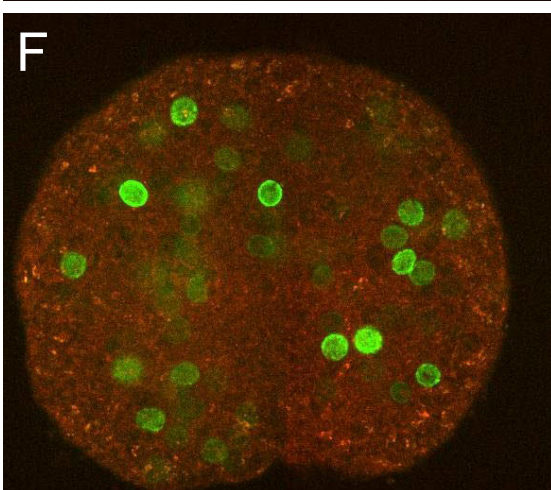
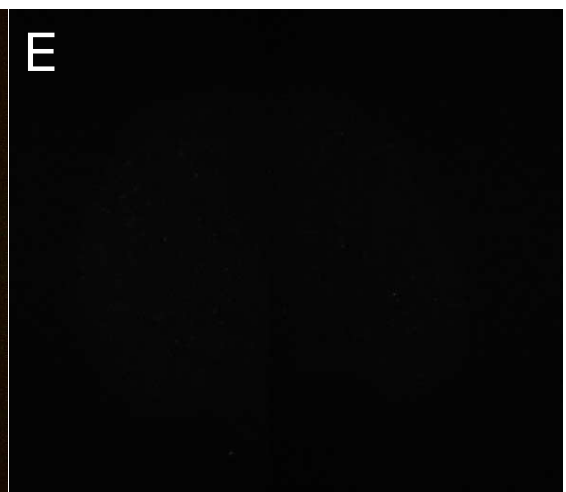
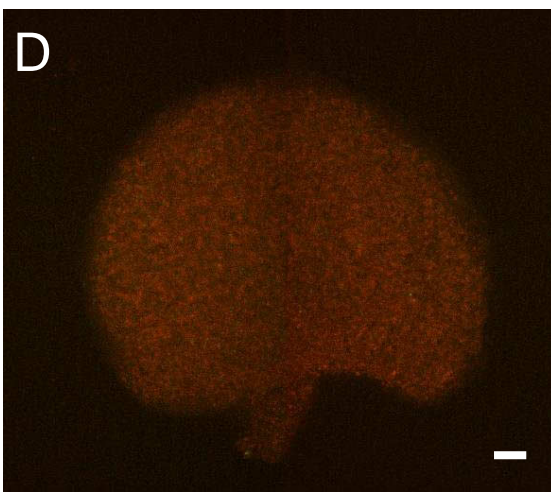
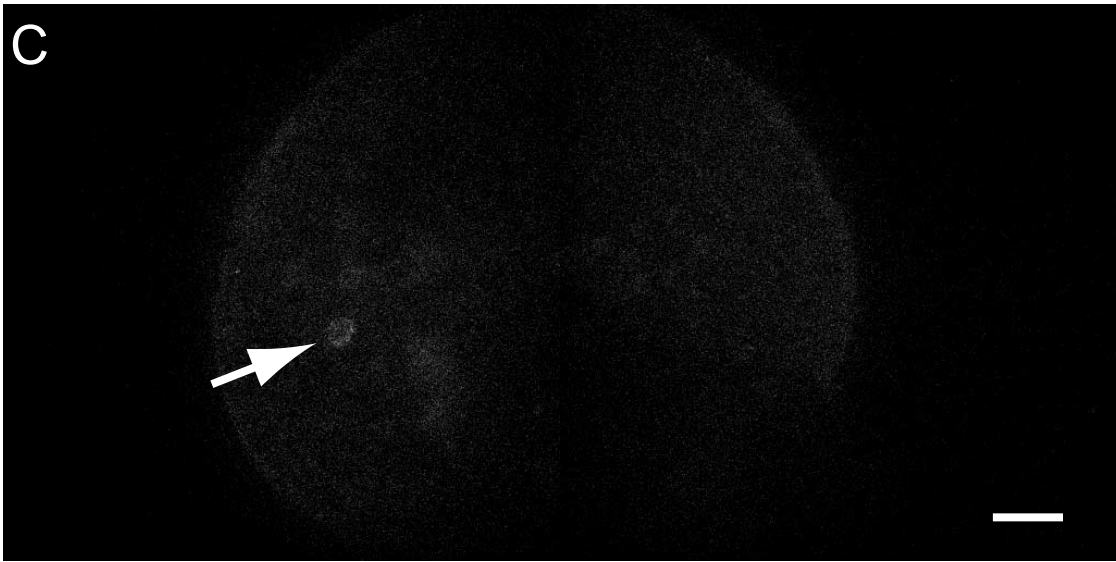
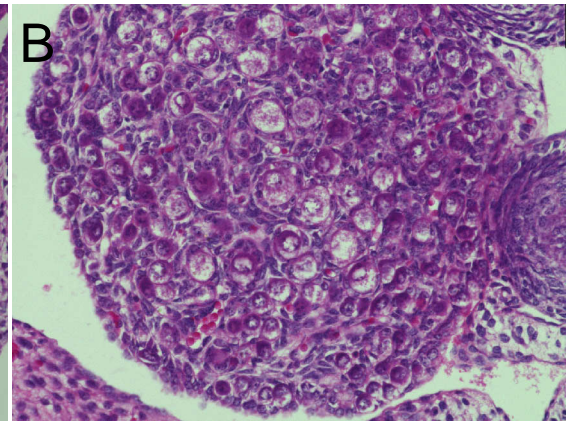
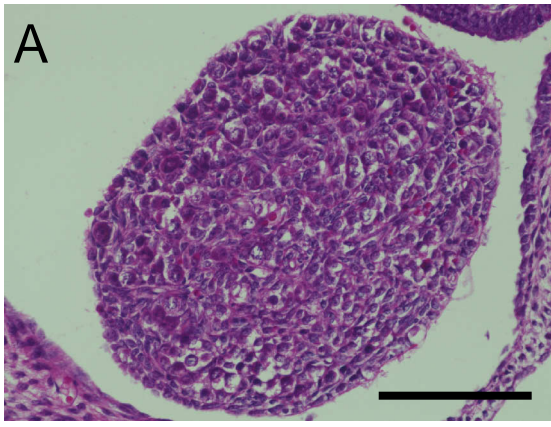


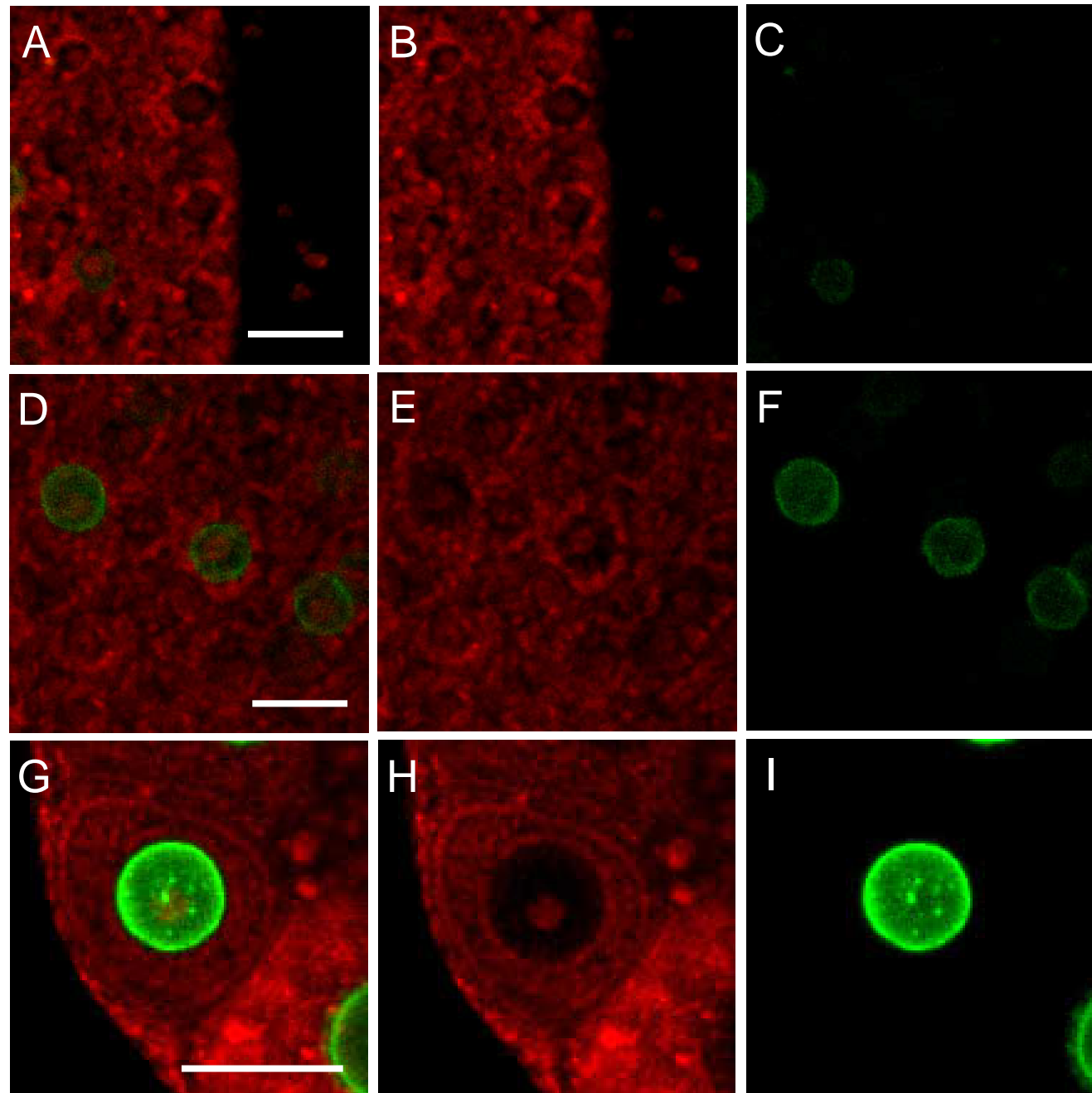
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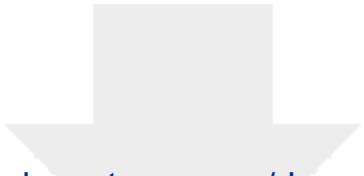


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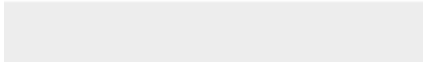



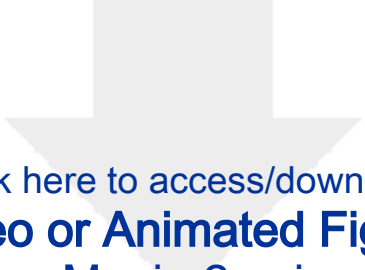




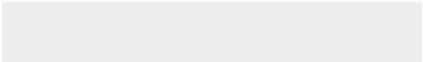



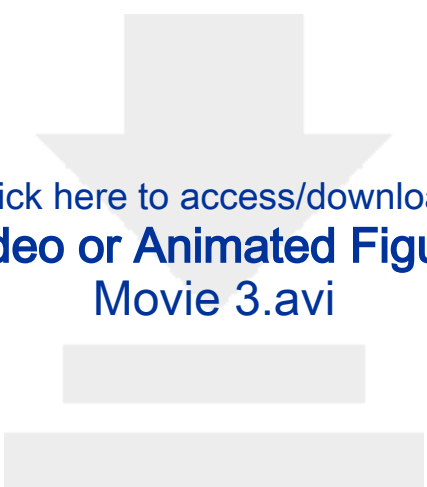
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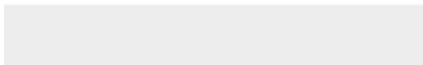
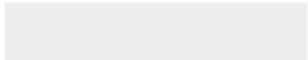


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microscope	objective lens	laser	exposure (ms)	Gain	time-interval	z-stack	others	Figures and Movies
CV1000	X10	488 nm: 30, 561 nm: 20	488 nm: 700, 561nm: 600	488 nm: 90, 561nm: 20	30 min	5 μ m		Figure 4C-G, Movie 1 and 2
BZ-X700	X20 (with collection collar)	40%	auto	X4	30 min	10 μ m	binning 3x3	Movie 3
LSM710	X10	488 nm: 6%, 564 nm: 5%	camera speed: 4	488 nm: 850, 564 nm: 850	24 h	10 μ m	digital gain, 488 nm: 2, 564 nm:1	Figure 2, 3 and 5

ovary no.	number of total ovulated oocytes	number of MII oocytes
1	7	2
2	19	9
3	14	6
4	7	3
5	12	4
6	15	6
7	25	11
8	13	3
9	11	7
10	19	8
11	18	4
12	7	1

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Follicle stimulating hormone from human pituitary	SIGMA	F4021	
Lutenizing hormone from equine pituitary	SIGMA	L9773	
Penicillin-streptomycin solution	Wako Pure Chemical Industries	168-23191	
MEM a, GlutaMax, no nucleotides	Thermo Fisher	32561037	
Glass bottom dish	MatTek	P35G-0-10-C	35mm dish, No. 0 coverslip, 10mm glass diameter
Millicell cell culture insert	Merck Millipore	PICM0RG50	Diameter: 315 mm, pore size: 0.4 mm, material: hydrophilic PTTE
3.5cm cell culture dishes	greiner bio-one	627160	
50ml / centrifuge tube with triple seal cap	IWAKI	2345-050	
Low-profile disposable blades 819	Leica	14035838925	
LSM 710	Carl Zeiss		Confocal microscope
CellVoyager, CV1000	Yokogawa Electric Corporation		Time-lapse imaging
BZ-X700	KEYENCE		Time-lapse imaging



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Author(s):

Kouji Komatsu, Akira Iwase, Tomohiko Murase, Satoru Masubuchi

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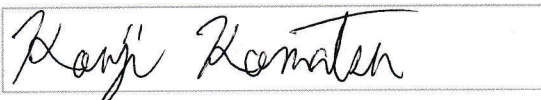
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Dear Dr. Bing Wu,

Thank you very much for the second reviews and the comments to our manuscript entitled “Ovarian Tissue Culture to Visualize Phenomena in mouse ovary” (JoVE57794). In the attached document, we described the changes made in response to the reviewer’s comments.

Editorial comments:

The manuscript has been modified and the updated manuscript, **57794_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We revised the manuscript after we got English proofreading again.

2. Please do not highlight notes for filming.

We deleted “Note” from highlighting for filming.

3. Please highlight more protocol steps for filming (less than 2.75 pages).

We added protocol steps, step 4-7, to highlight for filming.

4. Step 2.2: How to excise the ovaries? Using what? How to trim the tissues?

We revised this part.

“Excise the ovaries from 4-week-old female ICR mice and trim the tissues surrounding the ovary **using a scissors and a tweezers** under a stereoscopic microscope.” (line 113-114)

5. 2.2.1: What’s the size of the blade?

We added the information of the blade’s size in Table of Materials.

6. 3.2: How many ovary samples are placed in the 1 mL medium?

We revised the sentence to clear the number of samples in a drop.

“3.2 Place **single sliced specimens** into **one drop** of culture medium on cell culture inserts using tweezers (see step 3.1)

In addition to this point, we revised the volume of a drop, from “1 mL” to “0.5 μ L” (line 130)

7. 5.1: Please specify the imaging conditions.

We showed the conditions of time-lapse imaging in Table 1, so we added the word, “Table 1” (line 159). Furthermore, we modified Table 1 to clear the condition under which each figure and movie was captured.

8. 5.1.1: What laser intensities and exposure time are used?

We showed the conditions of time-lapse imaging in Table 1, so we added the word, “Table 1” (line 163). Furthermore, we modified Table 1 to clear the condition under which each figure and movie was captured.

9. 5.1.2: How to compare?

We revised this part.

“5.1.2. Compare follicle growth under each differing culture conditions **by measuring follicle areas in images of control samples at 24-h intervals and in time-lapse imaging samples. (See step 6).**”

10. 5.2: How to capture the images?

We revised this part

“5.2. Capture images at 30-min intervals using the time-lapse imaging system under the determined condition (Table 1).”

11. 7.3: Please specify the settings.

We added the information of setting condition of each figure and movie in Table 1.

Sincerely,

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