

Video Article

Three-dimensional Reconstruction of the Vascular Architecture of the Passive CLARITY-cleared Mouse Ovary

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Abstract

The ovary is the main organ of the female reproductive system and is essential for the production of female gametes and for controlling the endocrine system, but the complex structural relationships and three-dimensional (3D) vasculature architectures of the ovary are not well described. In order to visualize the 3D connections and architecture of blood vessels in the intact ovary, the first important step is to make the ovary optically clear. In order to avoid tissue shrinkage, we used the hydrogel fixation-based passive CLARITY (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/In situ-hybridization-compatible Tissue Hydrogel) protocol method to clear an intact ovary. Immunostaining, advanced multiphoton confocal microscopy, and 3D image-reconstructions were then used for the visualization of ovarian vessels and follicular capillaries. Using this approach, we showed a significant positive correlation ($P < 0.01$) between the length of the follicular capillaries and volume of the follicular wall.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56141/>

Introduction

The follicle is the fundamental structural and functional unit of the ovary, and its development is highly related to the vasculature within the ovary. Blood vessels supply nutrition and hormones to the follicles and thus play important roles in the growth and maturation of follicles¹.

A combination of technologies, including selective blood vessel markers, transgenic mouse models, and pharmaceutical development, have increased our knowledge about ovarian vascular networks, angiogenesis, and the function of blood vessels in folliculogenesis. The ovary is known as an active organ because it remodels various tissues and vascular networks during folliculogenesis and ovulation. Such active remodeling in the size and structure of vessels is required for the biological function of developing and recruiting follicles.

Traditional histological and histomorphometric methods using ovarian sections and immunolabeling of blood vessels are limited to two-dimensional (2D) images². With the development of three-dimensional (3D) reconstruction technologies, 2D images of tissue slices can be overlapped to make a 3D structure, but this method still has some limitations — sectioning of the tissue can destroy the microstructures, some parts of the tissue are often missing, and significant labor is involved in making 3D reconstructions from images obtained from slices. Whole-tissue 3D imaging with confocal microscopy can overcome many of these limitations, but these methods are limited to the evaluation of angiogenesis in the embryonic ovary³. Using whole tissue clearing methods such as CLARITY⁴ can increase the visualized volume so as to solve these problems in postnatal and adult ovaries, and such methods provide optical clearance of the ovary without any structural deformations. Imaging of the 3D architecture of the intact ovary provides an accurate image database for image analysis software, such as the Imaris software package used in this work.

Remodeling of the ovary throughout adulthood is part of a dynamic physiological system, and this makes the ovary an excellent model for investigations into the regulation of angiogenesis. Furthermore, evaluating the role of ovarian blood vessels in pathologic conditions of the female reproductive system such as polycystic ovary syndrome or ovarian cancers can be studied through whole ovarian tissue imaging. The development of the passive CLARITY method and the use of advanced image analysis software have provided detailed spatial information on the relationships between blood vessels and ovarian structures such as follicles.

Protocol

All procedures involving animal subjects followed the guidelines of the Animal Ethics Committee at Shanghai Medical College, Fudan University (Approval number 20160225-013).

1. Preparation of the Transparent Mouse Ovary

1. Preparation of the solutions

1. Prepare phosphate-buffered saline (PBS) solution (1 M, pH 7.6) with 0.1% Triton X-100 (PBST). To make 1 L of 10x PBS stock solution, mix 87 g of NaCl, 3.1 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, and 28.7 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$. Add dH_2O to 1 L and stir ~2 h. Adjust to pH 7.4 with 1 N NaOH and store at room temperature. Dilute this 10x stock solution by 1/10 using dH_2O and then make 0.1% Triton X-100 in PBS.
2. Prepare the hydrogel solution (pH 7.5) by mixings 4% (wt/vol) paraformaldehyde (PFA) solution, 4% (wt/vol) acrylamide, 0.05% (wt/vol) bisacrylamide, and 0.25% (wt/vol) VA-044 initiator in double-distilled water on ice.
3. Prepare the clearing solution (pH 8.5) by mixing 200 mM sodium borate buffer containing 4% (wt/vol) sodium dodecyl sulfate (SDS) in double-distilled water.

2. Transcardial perfusion and ovary preparation

1. Keep all solutions on ice during all procedures.
2. Anesthetize adult female wild type C57BL/6 mouse with an intraperitoneal injection of pentobarbital (0.35 mg/kg) solution. Once it shows no toe-pinch response, the mouse is properly anesthetized.
3. Use sticky tape to fix the mouse with limbs stretched out on a flat foam board.
4. Expose the heart by an incision on the xiphoid-process through the chest, skin, and bones with scissors.
5. Make an incision into the animal's right atrium, and then perfuse the left ventricle with 20 mL of ice-cold 1x PBS at 10 mL/min.
6. Perfuse the animal with at least 20 mL of hydrogel solution at 10 mL/min.
7. Incise the skin over the abdomen along the medioventral line with scissors, which exposes the whole enterocoelia, and remove the intestines. Collect the ovaries as well as the uterus to maintain the integrity of ovarian morphology.
8. Remove the fat tissues around the ovary under a microscope.

3. Degassing

1. Place the ovaries in 10 mL hydrogel solution at 4 °C for fixation for 3 days.
2. Transfer the ovary into a 5-mL tube and fill the tube with hydrogel solution. Stretch parafilm over the top of the tube and then wrap parafilm around the neck of the tube.
Caution: Ensure that there are no bubbles between the parafilm and the hydrogel solution.
3. Place the tube on a shaker in the incubator at 37 °C for a maximum of 3 h to allow the hydrogel to polymerize.
4. Remove the ovary from the gel using a spatula and carefully remove the excess gel from around the ovary by tissue paper. Wash the ovary four times with 1x PBS.

4. Passive clearing

1. Submerge the ovary in 10 mL of the clearing solution. Gently shake at 80 rpm at 37 °C to start the clearing process.
2. In the first week, change the solution every 3 days. After one week, refresh the clearing solution once a week until the ovary becomes clear. Usually this process takes about 2-3 weeks.
NOTE: The ovary can be directly immunostained or can be stored in sodium azide (0.02%) in PBS at 4 °C for several weeks before immunostaining.

2. Immunostaining

1. Wash the ovary twice in PBST on the day 1 at 37 °C on a shaker.
2. Incubate the ovary on a shaker in primary antibodies/PBST solution (**Table 1**) on day 2-3 at 37 °C. In the work presented here, the primary antibodies were platelet-endothelial cell adhesion molecule 1 (CD31) and tyrosine hydroxylase diluted at 1:10 and 1:50 in PBST, respectively.
Caution: If two or more kinds of primary antibody are used, make sure there are different animal sources.
3. Wash off the primary antibodies with PBST buffer at 37 °C on day 4 on a shaker.
4. Incubate the ovary with the desired secondary antibodies in PBST on day 5-6 at 37 °C on a shaker. In the work presented here, the secondary antibodies were Alexa Flour 488 for tyrosine hydroxylase and Alexa Flour 594 for CD31 diluted at 1:50 in PBST.
5. Wash off the secondary antibodies with PBST buffer at 37 °C on day 7 on a shaker.
6. Transfer the tissue into refractive index matching solution for refractive index homogenization on day 8. Put the tissue on a paper with gridlines to check its visual clarity by checking whether the gridlines can be seen. Once the tissue has been fully clarified, it can be imaged.
7. Make a putty cylinder that could just surrounds the tissue, and then shape it into a horseshoe shape and press the outer ridge tightly onto a glass slide in order to make a sealed space. The height of the putty should be a little taller than the ovary.
8. Place the clarified ovary carefully into the center of the putty and add refractive index matching solution. Put a glass-bottom dish over the putty tightly and use putty to fix the dish.

3. Confocal Microscopy

1. For imaging with a confocal microscope, in the "Ni-E" panel choose the lens (in this case water immersion 25X objective lens, 1.1-NA, 2 mm-WD) with the proper working distance (in this case 2.0 mm).

2. In the "Acquisition" panel, select the number of channels. Here, use three channels.
 1. First, use the "eye port" tab and the XY controller joystick to move the tissue to the center of field by checking through the microscope eyepieces.
 2. After turning off the shutter light, click the "live" tab to adjust the "laser power", "HV (Gain)", and "offset" for each channel separately. Higher laser power and HV can lead to higher signals, and higher offset can reduce the background noise.
 3. Select the proper "Scan size" and "Count". Use a scan size of 1024 x 1024 μm^2 and a count of 4 to 8.
3. After setting the image quality for defining the depth of the tissue in the panel "Z intensity correction", and after locating the lens on the top-most layer of the ovary (in the center of the tissue) and adjusting the acquisition for the top layer, click the "plus" button to define the top layer.
 1. Move the lens down and set the appropriate HV and laser power for the bottom of the tissue and add the lowest layer information into the Z-intensity correction table. Click on "To ND" to synchronize the setting with the "ND acquisition" panel.
4. In the "ND acquisition" panel, first set the number of scanning steps. Then, tick the tabs "Z" and "Large Image".
 1. Go to the "Scan large image" window to define the size of the field according to the border of the tissue with the help of tissue visualization through the eyepieces. Go back to the "ND acquisition" panel to input the size of the scanning area ("fields") and choose the overlap between 10% and 15%.
 2. Click "Run Z correction", not "Run now", to automatically set the proper HV, laser power, and offset for each layer, and wait for the image to process.

4. 3D Reconstruction of Individual Follicles and Their Vasculatures

1. First, use ImageJ⁵ to open the original NIS file. Click the "Image" button and choose the "Color" and the "Split Channels". Then, click "File" and separately save the channels as tiff file series by using the "Image Sequences" option in "Save as".
 2. Use Imaris to open one of the tiff file series. Click the "Edit" button and choose "Add channels" to add the rest of the channels. The name and color of the channel can be changed in the "Display Adjustment" tabs. The thickness of the tissue might need to be corrected in the "Image Properties" panel in the drop-down list under "Edit".
 3. For the 3D cropping of the final images and removal of excess parts, click the "Edit" button and choose "Crop 3D". The size of the field can be adjusted by dragging the border.
 4. To reconstruct the vessel signals with the Filaments algorithm in the Surpass panel, click the "Filaments" button to create a new Filament and click next.
 1. In order to define the largest and the thinnest diameter, go to the "Slice" panel and find the tracing vessel. The distance will be automatically shown on the "Measure" panel by selecting two points at the maximum width of the vessel.
 2. Go back to the "Surpass" panel and input the measured width and click next.
 5. Adjust the starting and seed point threshold. In the "Camera" panel, choose "Select". If some of the automatically produced spheres need to be removed, press shift and click on the point. By choosing "Navigate" and moving the mouse, the structure can be rotated to ensure that all the correct points have been retained, and then click next.
 6. Choose the highest threshold for the local contrast and click next. Do not select "Detect Spines", and continue to finalize the reconstruction of the blood vessel cylinder. The color can be edited by clicking the "Color" tab. The statistical data can be extracted in the "Statistic" panel of the reconstructed Filament. Excess parts can be removed in the "Edit" panel.
- NOTE: Other algorithms can be used for measuring the volume of total follicles or follicular walls. In the work presented here, the oocytes were marked by the autofluorescence of both secondary antibodies. Because of the large amount of data, a workstation server was used for the data analysis.

5. Statistical Analysis

NOTE: Image analysis data are presented as means $\pm$ standard errors.

1. Perform comparisons between the follicles using independent sample t-test, and use Pearson's correlation test to compare correlation coefficients of follicular wall and capillary length. Set a value of $P < 0.05$ as the limit for statistical significance.

Representative Results

We adapted the passive CLARITY method into a quick and simple method for passive ovary clearing while preserving the follicular and vascular architecture and obtaining the highest fluorescent signal from labeled markers of vessels and follicles. The 3D architecture of the follicular vasculature was determined by immunostaining for CD31, a marker for endothelial cells⁶. CD31 staining in the ovaries of adult mice was traced using the Filament algorithm and showed interrelationships between follicular capillaries and primary and secondary follicles (**Figure 1**).

The Spot transformation and Surface algorithms were used to detect the tyrosine hydroxylase-immunostained oocytes and granulosa and thecal layers and to calculate the total volume of follicles^{7,8}. Because of the compaction of the follicles, some parts of cropped follicles were identified semi-manually. After Spot transformation of the individual follicular volume and oocytes and Surface reconstruction of the follicular wall layers and Filament reconstruction of the vessels, clear relationships between individual follicular vasculatures and the other measured indices, especially granulosa and thecal layer volumes, were observed (**Figure 2**).

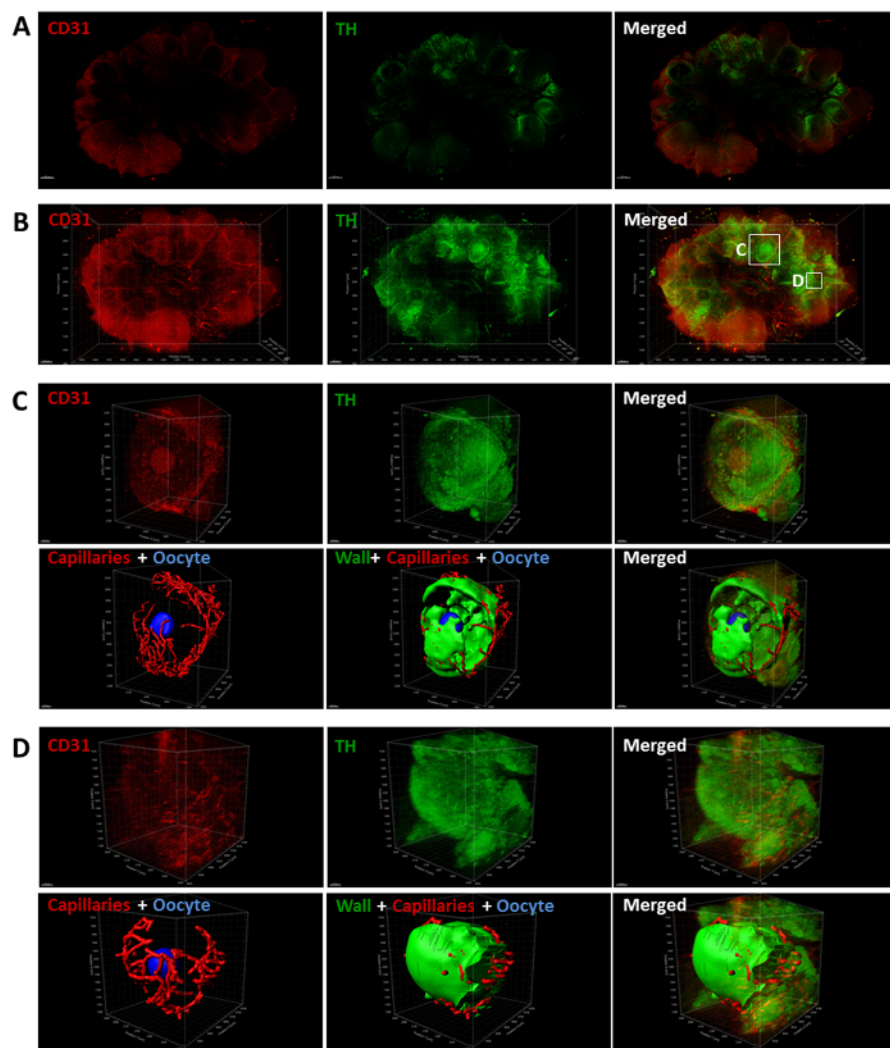


Figure 1: High-resolution imaging of an intact adult mouse ovary after clearing with the adapted passive CLARITY protocol. (A-B) Sections and XYZ large-scan acquisition. Three dimensional (3D) cropped secondary (C) and primary (D) follicles (top row) and 3D reconstructed capillaries, oocytes, and granulosa/thecal cells of the same follicle (bottom row) are shown as identified by the Filament, Surface, and Spot transformation algorithms. Scale bars = 200 μm (A-B), 50 μm (C), 30 μm (D). [Please click here to view a larger version of this figure.](#)

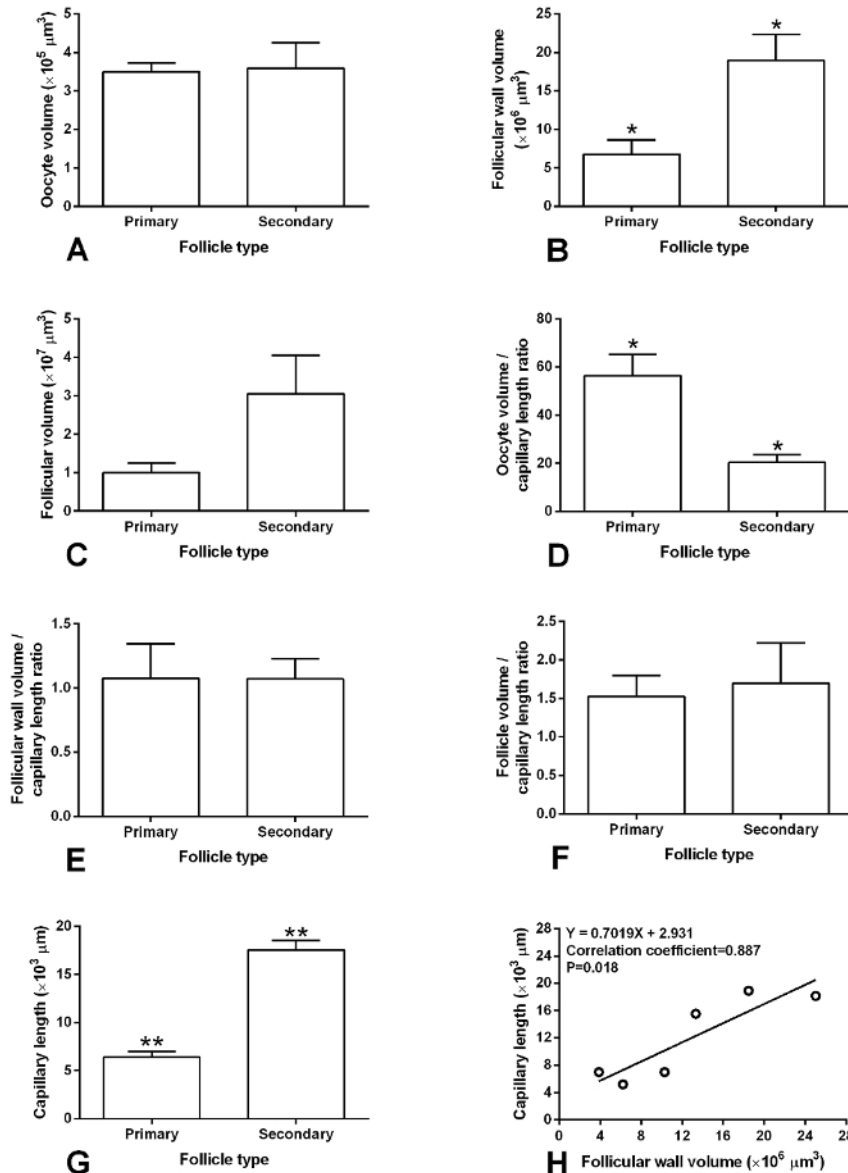


Figure 2: Comparative indices of the relationships between primary and secondary ovarian follicles and their vasculatures. The oocyte volume (A) was extracted after 3D reconstructions of follicular volume and oocytes in individual follicles as determined by the Spot transformation algorithm, and the follicular wall volume (B) was determined by the Surface algorithm and the capillaries were traced by the Filament algorithm. The follicular volume (C), the oocyte volume/capillary length ratio (D), the follicular wall volume/capillary length ratio (E), the follicular volume/capillary length ratio (F), the capillary length (H), and the correlation of capillary length and follicular wall volume (G) were calculated using the appropriate tools in the software package. Error bars represent the standard errors of the means. $n = 6$; * $P < 0.05$; ** $P < 0.01$. [Please click here to view a larger version of this figure.](#)

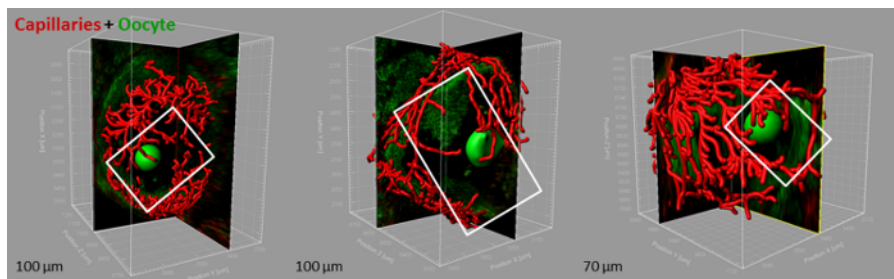


Figure 3: Three-dimensional reconstruction of the capillaries in secondary follicles of the mouse ovary. This reveals a hollow space above the oocyte side of the follicles (white squares show the borders of the space). [Please click here to view a larger version of this figure.](#)

Discussion

In the current study, we present 3D imaging to evaluate the relationships between capillaries and individual growing follicles. In our previous work using the same protocol⁹, we studied the roles of large vasculature, interactions between follicles, and the location of follicles in intact mouse ovaries. The passive CLARITY approach allowed us to study micro- and macro-vasculatures, folliculogenesis, and the interrelationships between the corpora lutea and follicles as well as to reconstruct the ovarian architecture at different developmental stages.

To obtain 3D image data of intact tissues, the clearing process is the first important step. There are two main strategies — dehydration methods and hydrophilic reagent-based methods, such as passive CLARITY¹⁰. Because dehydration methods often lead to a decrease in fluorescent signals and often produce toxic organic solution waste, we have focused our attention on simplifying some steps of the passive CLARITY method for use with various tissue types.

The first critical procedure in the clearing process is the transcardial perfusion. During the perfusion, heart pumping plays an important role in the whole-body circulation of PBS and hydrogel solution, which can flush out the blood so as to decrease the background fluorescence. Thus, the perfusion surgery should be performed accurately and quickly to avoid the premature death of the animal before the hydrogel is completely distributed throughout the animal. All agents must be kept on ice during the entire experiment, as mentioned by Liang *et al.*¹¹, or they will begin to polymerize before the perfusion is complete. The next important step is removing the hydrogel, and it is necessary to remove as much excess gel as possible because it will retain antibodies. Once the tissue is cleared, it should be removed from the clearing solution and stored in PBS at 4 °C because too much time in the clearing solution will cause too much protein to be lost. In the confocal microscopy imaging, the Z-settings should always be set before the X-Y range. If the X-Y range is set down first, the Z-settings cannot be synchronized to the entire X-Y field.

Passive CLARITY was first introduced by Yang *et al.*¹² In their protocol, they did not perform the perfusion step and instead degassed with nitrogen. In addition, their clearing solution did not include bisacrylamide in the hydrogel solution, they used 8% SDS in the clearing solution, and they used RIMS for refractive index matching media. This method was subsequently improved by increasing the SDS concentration¹³, adding the perfusion step and the nitrogen-free degassing procedure¹⁴, and combining the method with other clearing methods such as PARS-mPACT¹⁵. Here, we have further developed the protocol by combining transcardial perfusion and nitrogen-free degassing, decreasing the SDS in the clearing solution to 4%, and using FocusClear as the refractive index matching media, which provides better-quality microscopy images.

After passive CLARITY, confocal multiphoton microscopy and image analysis can be performed to obtain the intact 3D architecture of the ovarian follicular vasculature. This protocol transforms a non-light-permeable intact ovary into an optically transparent hydrogel-hybridized nanoporous form that is appropriate for penetration by macromolecules such as antibodies¹⁶. Unlike classical 2D sectional histological analyses, passive CLARITY and staining with specific markers allows the 3D mapping of relationships among capillaries and individual growing follicles.

In adult tissues, active vascular remodeling and angiogenesis mainly occurs during pathological processes such as wound healing, fracture repair, tumor and metastasis formations, retinopathies, fibroses, and rheumatoid arthritis¹⁷. However, cyclical changes during folliculogenesis represent a unique capacity for vasculature remodeling. After formation of the antrum in secondary follicles, follicular fluid fills it and surrounds the oocyte, which provides a suitable microenvironment for oocyte maturation and follicular development¹⁸. Previous 2D imaging studies showed that ovarian follicles are surrounded by a network of capillaries in the theca interna¹⁹. The granulosa layer is nonvascular, and each follicle is surrounded by its own capillaries. Consistent with the essential role of CD31 in vasculogenesis⁶, we found an increase in capillary length during folliculogenesis in the transition from primary to secondary follicles (**Figures 2G and 2H**). Instead of a random distribution of capillaries around the individual primary or secondary follicles, 3D reconstruction of capillaries demonstrated that capillary branches distributed in follicles were detectable in larger follicles, and in most cases these capillary branches formed a hollow space (**Figure 3**).

One major concern is that the adapted method takes a relatively long time to clear the ovary, but the long time is required for the best clearing effect. To save some time, we recommend collecting all of the needed samples at one time and clearing them together because the cleared tissues can be stored in PBS and 0.02% sodium azide at 4 °C for several months. Another limitation is the working distance of the confocal microscope. Higher-magnification objective lenses have shorter working distances, which could be an obstacle for imaging of thick samples.

Several protocols have been introduced for the imaging of transparent and intact tissues, including BABB²⁰, Scale²¹, 3DISCO²², ClearT²³, SeeDB²⁴, CLARITY¹⁶, passive CLARITY (with⁴ or without hydrogel²⁵), PACT¹², CUBIC^{26,27}, FASTClear²⁸, and FACT²⁹, but only three of them have been used for whole ovarian tissue clearing and evaluation^{9,30,31}. SeeDB³⁰ and ScaleA2³¹ have been introduced for imaging of whole ovarian tissues, but these protocols only allow a single immunostaining step. Passive CLARITY, however, allows for multiple protein-labeling reactions, as shown in the present study and in our previous study⁹.

In conclusion, our adapted protocol for determining the 3D structural relationship between follicular layers and blood vessels using whole clarified ovarian tissue provides a new approach for studying angiogenesis in different ovarian diseases such as polycystic ovary syndrome and ovarian cancers.

Disclosures

The authors have nothing to disclose.

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