Research Article

C-ECi: a CUBIC-ECi combined clearing method for three-dimensional follicular content analysis in the fish ovary†

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†Grant Support: This work was supported by the TEFOR project (Agence National de la Recherche, ANR-II-INBS-0014) and the DYNAMO project (Agence National de la Recherche, ANR-18-CE20-0004).

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Received 11 March 2020; Editorial Decision 5 August 2020; Accepted 7 August 2020

Abstract
Deciphering mechanisms of oocyte development in the fish ovary still remain challenging, and a comprehensive overview of this process at the level of the organ is still needed. The recent development of optical tissue clearing methods has tremendously boosted the three-dimensional (3D) imaging of large size biological samples that are naturally opaque. However, no attempt of clearing on fish ovary that accumulates extremely high concentration of lipids within oocytes has been reported to date. To face with this ovarian-specific challenge, we combined two existing clearing methods, the nontoxic solvent-based ethyl cinnamate (ECi) method for efficient clearing and the Clear Unobstructed Brain Imaging Cocktails and Computational (CUBIC) method to enhance lipid removal and reduce nonspecific staining. The methyl green fluorescent dye was used to stain nuclei and delineate the follicular structures that include oocytes. Using this procedure (named CUBIC-ECi [C-ECi]), ovaries of both medaka and trout could be imaged in 3D and follicles analyzed. To our knowledge, this is the first procedure elaborated for clearing and imaging fish ovary in 3D. The C-ECi method thus provides an interesting tool for getting precise quantitative data on follicular content in fish ovary and promises to be useful for further developmental and morphological studies.

Summary Sentence
A modified ethylcinnamate-based clearing method allowing 3D imaging of fish ovary and analyzing follicular content.
Introduction

Although much effort has been made in recent years toward understanding oogenesis mechanisms at a cellular level in fish, we still lack a comprehensive overview of this dynamic process at the level of the organ. Tightly regulated networks of hormones, secreted factors, and intrinsic signaling pathways underlie the progression of each single oocyte throughout oogenesis and control the female fecundity [1–3]. In terms of timing, it however exist different dynamics of oogenesis in adult females, in relation with the different reproductive strategies that exist in fish [4]. So far, studies related to oogenesis dynamics in fish have mainly been based on follicular (i.e., oocytes and their surrounding somatic supporting cells) content analyses from ovarian 2D histological sections, which require extensive extrapolations of data [5]. Three-dimensional (3D) analyses of ovarian follicular contents would thus be a major milestone on the path toward a better comprehension of the oogenesis temporal dynamics in fish.

Techniques for 3D imaging of large biological samples that are naturally opaque have experienced major technical breakthroughs this past decade, including the development of numerous optical clearing methods to enhance tissue transparency and reduce light scattering [6]. These methods are either aqueous- or organic solvent-based methods and are grouped into four main classes, including the simple immersion methods, hyperhydrating methods, solvent-based methods, and tissue transformation methods [6, 7]. Simple immersion methods rely on the homogenization of refractive indexes of medium and tissues by using high-refractive index (hRI) aqueous solutions. These solutions are composed of the contrast agent iohexol (also called Histodenz), such as for the RI Matching Solutions (RIMS) or are sugar-based methods, such as for SeeDeepBrain (SeeDB) [8–10]. Methods of the second class are hyperhydrating solutions, such as the CUBIC analysis that is based on the use of the hyperhydrating aminoalcohols, urea, and removal of lipids with detergent [11]. Methods of the third class are tissue transformation methods, such as the emblematic Passive CLARITY Technique (PACT) that uses a hydrogel to stabilize the tissue structure while removing lipids with detergent [8]. In the fourth class, the 3D Imaging of Solvent-Cleared Organs method (3DISCO) allows to bypass clearing performance limitations of water-based clearing methods, while preserving fluorescence [12]. More recently, Klingberg et al. developed a new clearing method using the organic compound ethyl-3-phenylprop-2-enoate (ethyl cinnamate [ECi]) that is extremely efficient for clearing animal tissues, including mammals [13–15]. ECi is considered nontoxic according to the European directive 67/548/EWG and is a Food and Drug Administration-approved food flavor and additive for cosmetic products [16]. Some of these clearing methods were already used for clearing the whole mouse ovary and allowed successful 3D imaging [17–20]. However, no attempts on fish ovary, which contains large size (up to 1 mm) and lipid rich oocytes, have been reported with any of these clearing methods either in zebrafish, medaka, or any other model fish species.

We tested several of these clearing methods on the medaka ovary. The fluorescent methyl green (MG) nuclear dye was used to delineate ovarian follicles. Our study reports that combining the CUBIC and ECi clearing methods allows solving the fish ovarian-specific challenges and optimizing the 3D fluorescent imaging. With this protocol, named CUBIC-ECi (C-ECi), we used a specific objective compatible with solvents immersion, which allowed high-resolution 3D imaging of ovaries of both medaka and rainbow trout. Such methodology will help evaluating the number of ovarian follicles in the fish ovary and providing new information on regulatory mechanisms that underlies female fecundity.
Materials and methods

Ethical statements
All experimental procedures used in this study followed the recommendations of the French and European regulation on animal welfare. Fish rearing and handling were approved by the INRAE LPGP-Animal Care and Use Committee (N° Z-2015-30-VT and Z-2015-127-VT-MF) for medaka and by the INRAE PEIMA Institutional Animal Care and Use Ethical Committee for rainbow trout (B29-277-02).

Fish breeding and sample collection
Adult medaka (Oryzias latipes) females from the CAB strain were raised in the INRAE-LPGP fish facility at 26 °C under an artificial photoperiod (14 h light/10 h dark), during which the length of the reproductive cycle is of 24 h and eggs are daily spawned at the onset of the light. Female rainbow trout (Oncorhynchus mykiss) from an autumn-spawning strain were held under natural photoperiod until their first reproduction (2 years) in INRAE-PEIMA experimental fish facilities (Sizun, France). For ovary dissections, medaka (aged from 4 to 5 months) and trout (2.5 years old, 134 days after the first spawning) fishes were euthanized by immersion in a lethal dose of MS-222 solution at 30–50 and 400 mg/L, respectively. Ovary of both medaka and trout were fixed overnight at 4 °C in 4% paraformaldehyde (PFA) diluted in 0.01 M phosphate buffer saline pH 7.4 (PBS, Sigma-Aldrich P4417 Sigma-Aldrich (St. Louis, Missouri United-States)). Then, ovaries were washed in PBS and conserved at 4 °C in PBS + 0.5% (w/v) sodium azide (S2002, Sigma-Aldrich).

Optical tissue clearing protocols
A schematic overview of clearing experimental workflows is shown on Figure 1A and 2A.

Simple immersion. All steps were performed at room temperature on an agitator. High-refractive index (hRI) matching solutions were prepared as described in Yang et al. with some modifications [8]. A 60% sorbitol-based solution was prepared by dissolving 300 g of Sorbitol (Sigma-Aldrich S1876) in 400 mL of PBS:DMSO (50:50) (v/v). The RI was adjusted at 1.457 by addition of PBS:DMSO. Dilutions of this solution (1:6 and 11:15) were prepared to reach lower RI (1.353 and 1.40). To reach RI 1.49, 120 g of sorbitol was dissolved in 50 mL of PBS:DMSO. For RI 1.456, 13.32 g of Histodenz (Sigma-Aldrich D2158) was dissolved in 10 mL of PBS 0.02 M (Sigma-Aldrich P4417). For each of the solutions described previously, ovaries were immersed successively in 20% (v/v) for 8 h, 40% (v/v) for 16 h, 60% (v/v) for 8 h, 80% (v/v) for 16 h, and 100% (v/v) for 48 h.

iDISCO+. Protocol was conducted as described by Renier et al. with slight modifications [21]. All steps were performed at room temperature on an agitator except with dibenzyl ether. Samples were dehydrated in a 20–40–60–80–98% (v/v) methanol series containing 2% Tween20 for 12 h each. A final bath in 100% methanol was performed during at least 24 h. Ovaries were incubated successively in 66% dichloromethane (DCM, Sigma-Aldrich 270997)/33% methanol for 3 h and in 100% DCM twice during 15 min. Sample was then immersed at least 15 min in dibenzyl ether (DBE, Sigma-Aldrich 108014) for refractive index matching.

Figure 1. Assessment of the transparency of adult medaka ovaries treated with different clearing methods. Ovaries dissected from adult medaka were cleared with hRI immersion solutions, CUBIC, ECI, or iDISCO+ methods. From one to three samples were used in each condition. (A) A schematic overview of experimental workflows. (B) Uncleared ovary in PBS. (C–G) Ovaries cleared with the indicated methods, CUBIC, or iDISCO+. Transparency was assessed by the visualization of black squares. Dotted black line indicates the edge of ovaries after clearing. The solvent-based clearing methods ECI and iDISCO+ are the most efficient methods for clearing medaka ovary. (K) Comparison of the fluorescent recovery in depth after CUBIC and ECI methods. The maximal signal recovery in depth was obtained with the ECI method although an important intrafollicular background was observed. Square = 1×1 mm.

CUBIC. A protocol was conducted as described previously by Suzaki et al. with slight modifications [11]. Ovaries were incubated 3 h in 50% CUBIC-1/50% PBS at room temperature and then
transferred for 3 days in 100% CUBIC-1 at 37 °C on a rotating wheel. CUBIC-1 reagent is composed of 25% (w/w) urea (Sigma-Aldrich GE17-1319-01), 25% (w/w) N₂N₂N₂N₂-tetrakis(2-hydroxypropyl) ethylenediamine (Sigma-Aldrich 122262), and 15% (v/v) tritonX-100 (Sigma-Aldrich X-100) in distilled water. The CUBIC-1 reagent was renewed once after 24 h. Samples were rinsed three times in PBS at room temperature for 1 h and incubated for 2 days in CUBIC-2 reagent at 37 °C on a rotating wheel. CUBIC-2 solution was renewed after 24 h. CUBIC-2 is composed of 50% (w/w) sucrose (Sigma-Aldrich 59378), 25% (w/w) urea (Sigma-Aldrich GE17-1319-01), 10% (w/w) triethanolamine (Sigma-Aldrich 90279), and 0.1% (v/v) TritonX-100 in distilled water.

Ethyl cinnamate. ECi clearing was adapted from Klingberg et al. with few modifications [13]. All steps were performed at room temperature on an agitator. Samples were dehydrated in a dilution series of 20–40–60–80–98% (v/v) methanol/2% Tween20 for about 12 h each. Samples were then successively immersed in 100% methanol during 48 h and in 100% ECI for 3 h (without shaking). ECI solution was renewed and samples were placed on an agitator until complete clearing (i.e., from 24 h to several days).

CUBIC-ECi. Ovaries were successively incubated in 50% CUBIC-1/50% PBS for 3 h at room temperature and in 100% CUBIC-1 for 3 days at 37 °C on a rotating wheel, as for the CUBIC protocol. The CUBIC-1 reagent was renewed after 24 h. Then, samples were rinsed three times in PBS at room temperature for 1 h and processed according to the ECI protocol.

Nuclear staining with methyl green
Nuclear staining was performed before clearing steps in the ECI protocol and after CUBIC-1 in the CUBIC and C-ECi protocols (Figure 1A and 2A). Samples were incubated in MG in PBS/0.1% triton (pH7) or in TNT (TrisHCl 0.1 M, NaCl 0.15 M)/0.1% triton (pH4). MG was used at 80 or 8 μg/mL. Incubations were performed at 4 or 50 °C for 2.5 days. Samples were then washed overnight in PBS + 0.1% tween at room temperature.

Image acquisition
Bright-field macroscopic images were acquired with an upright Zeiss stereomicroscope with an Axiocam digital camera. Image post-processing (enhancement of brightness and contrast) was applied when necessary to homogenize the image background. Sample fluorescence imaging was performed with a Leica TCS SP8 laser scanning confocal microscope, using the 16x/0.6 IMM CORR VISIR HC FLUOTAR objective (ref. 15506533, Leica (Wetzlar, Germany)) dedicated to immersion acquisition mode within the Benzyl Alcohol/Benzyl Benzoate solvent-based solution. The MG fluorescent signal was detected through laser excitation at 638 nm, and fluorescent detection was performed by an internal photomultiplier. For clearing method comparisons, samples were imaged with the same settings, including no laser compensation in depth and no image post-processing. For 3D segmentation, whole samples were imaged with laser compensation and contrast enhancement was applied. Tiles of z-stack images were acquired and stitched into larger mosaics using the microscope software (11.72% overlap). Z-steps were fixed at 6 μm for all acquisitions. For medaka ovaries (about 3 mm thick) and because the working distance of the objective is limited (2.5 mm), dorsal and ventral faces were imaged separately. The overlap was of 1 mm. The ventral face of the ovary was fixed to a coverslip with epoxy glue (SADER 30610770) and placed in a glass Petri dish containing ECI. For dorsal and ventral face imaging, the coverslip was placed successively with the ovary up or down, respectively. For ventral face imaging, the coverslip was placed on a stainless steel washer as a spacer. A schematic overview of the mounting procedure is shown on Figure 3A. For each z-stack, a total volume of about 8 × 7 × 2 mm was acquired (voxel size: 1.8 × 1.8 × 6 μm) and generated about 5.5 GB of data. Acquisition of z-stack images took approximately 10 h for each face of medaka ovaries and 4 h for the trout ovary portion. All samples were imaged within the following week after staining and clearing.

Three-dimensional volume reconstruction and segmentation
Whole specimen image stacks acquired by confocal microscopy were treated with the Amira 2019.2 software Thermo Fischer Scientific (Waltham, Massachusetts, United States) on a 64-bit Windows 10 Pro computer equipped with a 2x Intel Xeon Silver 4110 (8 Core, 3.0GHz) processor, a Nvidia Geforce GTX 1080 graphic card and 384 Go of RAM. For medaka ovaries, Z-stacks of each face (ventral and dorsal) were stitched manually and merged by standard interpolation with the Amira software. To minimize the size of data, images were scaled to 0.3 (1:3) in X and Y. 3D follicle segmentation was performed semi-automatically in the project editor of Amira using a combination of different operation with systematic 3D interpretation. To binarize the nuclear signal, the image gradient (Canny–Deriche) was calculated and a Top-Hat was applied on resulting images to threshold nuclei. Images were inverted to visualize the internal part of follicles and connective tissues. Morphological operator like opening and hole filling was performed to improve follicles shapes. A watershed was applied to separate connected objects. To eliminate nonfollicles remaining structures, binary objects were filtered based on their size and shape. Only follicles with a spherical shape and an equivalent diameter above 50 μm were kept. Resulting 3D reconstruction of follicles was generated with Amira’s “Volume rendering” visualization module. Volumes of all segmented follicles were exported and equivalent diameters were calculated and corrected with the correction factor to compensate the volume shrinkage due to sample clearing. For 2D segmentation analysis, equivalent diameters were extracted from each image plane. For 3D volume reconstructions, follicles were colorized by diameter range, based on their corrected size.

Statistics
Statistical analyses were performed by using RStudio Version 1.1.463. P-values were calculated by using a Mann–Whitney test or a nonparametric Wilcoxon signed-rank test for indicating significant differences between samples.

Results and discussions
The solvent-based ECI method efficiently clears the medaka ovary
We first assessed the clearing efficiency on medaka ovary of various hRI simple immersion solutions, of the hyperhydrating CUBIC method and of solvent-based methods such as ECI or iDISCO+ (Figure 1A). For hRI simple immersion, samples were treated with hRI matching solutions adjusted to increasing refractive index, from 1.353 to 1.49. The apparent transparency in each condition was compared to that of an opaque uncleared ovary. With the different
Combining CUBIC and ECI methods efficiently improve follicular staining in the whole medaka ovary. Medaka ovaries were cleared following the ECI procedure (ECi) with or without a pretreatment with the aqueous reagent of the CUBIC method (C-ECi). The outlines of follicles were detected with the fluorescent MG nuclear dye using different conditions of pH and temperature. [A] Clearing protocols timelines. [B] Representative images of the MG fluorescent signal (in green) on XY planes at 1000 μm depth acquired with the same settings and from ovaries treated with ECi or C-ECi. Three samples were used in each condition. [C] Quantification of the signal contrast obtained with MG staining. The ratio signal/background was measured in different regions of interest (ROI) on sections. Violin plots are displayed on graphs for ECi (in blue) and C-ECi (in purple) treated samples with variations in the different conditions of temperature and pH. [D] The apparent ovarian surface areas were measured from brightfield images (black dotted lines) before and after clearing with ECi (n = 7) and C-ECi (n = 12). The tissue shrinkage is significant after C-ECi clearing (Wilcoxon test, \( P = 0.002516 \)). Data are presented as means ± SD. Square = 1 x 1 mm; scale bars: 250 μm. hRI solutions, samples displayed only a tiny increase of transparency that was maximal at RI 1.49 (Figure 1B–G). When treated with CUBIC (RI 1.48), transparency was significantly increased (Figure 1B and H). The higher transparency was obtained with the solvent-based ECI (RI 1.538) and iDISCO+ (RI 1.561) methods (Figure 1B, I, and J).

We then assessed the optical penetration in depth of confocal imaging after clearing with either CUBIC or ECI methods. Nuclei, including those of the theca and granulosa cells lining each oocyte, were stained with the MG far-red fluorescent nuclear dye [22]. Whole samples were imaged by confocal microscopy with no signal compensation in depth and no image postprocessing (Figure 1K). XZ orthoslices of the stacks showed a limited signal recovery in depth with CUBIC (<1 mm), whereas the signal recovery in depth was much higher with ECI (up to 2.5 mm), corresponding to the working distance of the objective. However, we observed a
was obtained with the solvent-based iDISCO [17], ScaleA2 [18], and CUBIC [23], but higher clearing efficiency for fetal and adult ovaries with the aqueous-based methods CLARITY and CUBIC. Similarly, previous studies in mouse reported the clearing of ovaries than aqueous-based methods (such as simple immersions or the ECi method).

A strong intrafollicular fluorescent background was observed with solvent-based methods. However, we must note that, at a microscopic level, this background is more appropriate for clearing fish ovaries than aqueous-based clearing methods, employing the highest RI matching solvent. It is thus even more consistent that solvent-based clearing methods, employing the highest RI matching solvent, are more appropriated for clearing fish ovaries than aqueous-based methods. Nevertheless, any homogeneous nonspecific intracytoplasmic background in EGi-treated samples, whereas no background was detected in CUBIC-treated samples (Figure 1K, stars). Similar background was observed in ovaries stained with a less concentrated MG solution (8 μg/mL, data not shown).

These results show that, at a macroscopic level, solvent-based methods (such as iDISCO+ or EGi) enable better clearing of medaka ovary than aqueous-based methods (such as simple immersions or CUBIC). Similarly, previous studies in mouse reported the clearing of fetal and adult ovaries with the aqueous-based methods CLARITY [17], ScaleA2 [18], and CUBIC [23], but higher clearing efficiency was obtained with the solvent-based iDISCO+ method [20]. The lipid-content is markedly higher in fish ovary than in mouse ovary, since fish oocytes exhibit higher lipid supply and larger size due to their telolecithal nature. It is thus even more consistent that solvent-based clearing methods, employing the highest RI matching solvents, are more appropriated for clearing fish ovaries than aqueous-based methods. However, we must note that, at a microscopic level, a strong intrafollicular fluorescent background was observed with the EGi method.

**Optimization of the EGi method**

Based on the assumption that the intrafollicular background was due to remaining lipids in oocytes after EGi treatment, we tested whether additional lipid removal may improve the staining specificity. Entire ovaries were thus incubated in the aqueous reagent 1 of the CUBIC clearing method (CUBIC-1) prior MG staining and EGi clearing [11]. Furthermore, we tested whether different MG clearing conditions may also contribute to reduce the background [24–26]. CUBIC-1 preincubated ovaries (C-EGi protocol) and not preincubated ovaries (EGi native protocol) were thus processed for MG staining at two different temperatures (4 and 50 °C) and pH (pH 4 and pH 7, Figure 2A). XY plane at 1000 μm depth from z-stacks of the different cleared samples were compared (Figure 2B). Follicles of different sizes were easily distinguishable in each condition, except with EGi at 50 °C-pH 4, where almost no fluorescent signal was recovered. Follicles appeared well packed in almost all conditions except at 4 °C-pH 4, where follicles appeared distorted and slightly dissociated. The fluorescence ratio signal/background was significantly increased with the C-EGi protocol and the higher ratio was obtained at 50 °C-pH 7 that offers the best signal contrast (Figure 2C).

To evaluate the effect of C-EGi clearing on the ovarian size, the apparent surface of the ovaries was measured on macroscopic images before and after EGi or C-EGi methods (Figure 2D). Almost no size modification was observed with EGi alone, whereas the apparent ovarian size was significantly reduced after C-EGi treatment (S2), compared to the ovarian size before treatment (S1):

\[
S_1 = 26.01 \pm 5.54 \text{ mm}^2
\]

\[
S_2 = 20.51 \pm 4.10 \text{ mm}^2.
\]

The shrinkage of ovaries treated with C-EGi were therefore of about 21%. We used the apparent ovarian surfaces measured before and after C-EGi treatment (S1 and S2, respectively) in order to calculate a correction factor and estimate the real diameter of follicles. We assumed that the ovarian shrinkage was due to the reduction of follicles size and that the reduction rate was independent of the follicular size, as observed after dehydration of isolated follicles of various sizes [27]. The correction factor (corr) was calculated as follows:

\[
corr = \frac{D_1}{D_2} = \frac{R_1}{R_2} = \sqrt{\frac{\pi R_1^2}{\pi R_2^2}} \approx \sqrt{\frac{S_1}{S_2}} = 1.12.
\]

This correction factor was applied to all measured follicular diameters in further analyses.

Here, we showed that combining CUBIC and EGi methods significantly reduces intrafollicular fluorescent background of MG staining in medaka ovary, likely by increasing delipidation of oocytes, and that 50 °C-pH 7 staining conditions significantly improve the staining. In a previous study, McKey et al. successfully used iDISCO for clearing and imaging the mouse ovary [20]. Interestingly, in this latter, a CUBIC treatment was added at the end of the protocol to allow immersion of the objective in an aqueous noncorrosive solution. By contrast, in the C-EGi method, the CUBIC treatment was performed at the beginning of the protocol in order to reduce the MG background, while increasing the clearing efficiency, and image acquisitions were performed by immersion within the EGi, using a dedicated immersion objective that resists to corrosive solvents. Anyway, we cannot exclude that similar results could also be obtained by using the solvent-based iDISCO method alone, since this later already includes a delipidation step with the organic dichloromethane reagent (DCM) [21, 28]. Although the iDISCO
Figure 4. 3D follicular content analysis in the adult medaka ovary. Medaka ovaries were processed through MG staining, C-ECi clearing, 3D imaging, and 3D follicular segmentation. (A) Ventral and dorsal views of a 3D reconstruction of a whole ovary merged with 3D-segmented follicles. (B) XY-planes at increasing depths showing image data (top panels) and image data merged with segmented follicles (bottom panels). Only few follicles were not detected (arrows). (C)
Assessment of 3D imaging and 3D segmentation of a C-ECi cleared medaka ovary

An ovary collected from an adult medaka female was stained with MG at 50 °C pH 7, cleared with the C-ECi protocol and imaged on both faces (dorsal an ventral) by confocal microscopy in immersion mode (Figure 3A). After stitching in Z, the final stack comprised 480 XY-image planes and the whole volume was reconstructed (Figure 3B). All follicles were detectable and no loss of resolution was observed throughout the sample (Supplementary Video S1). The final stack was used for 3D segmentation and analysis of ovarian follicles, using a computational semi-automatic 3D segmentation procedure. Follicles of various sizes were segmented and reconstructed as shown on the 3D views (Figure 4A). Only few follicles were not detected as shown on XY-planes (Figure 4B, arrows). To assess the gain of a 3D analysis of follicles, compared to a 2D analysis, the follicular diameters were extracted from the 3D segmented image data of the whole ovary and compared to those extracted from the corresponding 480 XY-image planes. The correction factor was applied to all diameter values. The number of follicles and the size distribution were analyzed in both cases. We obtained a total of 1323 follicles (ranging from 50 to 963 μm diameter) in the whole ovary, while using 2D-image data the total number of follicles measured on XY-planes ranged from 1 to 164, with an average of 144 follicles on planes between 700 and 1700 μm depth (representing a total of 167 XY-planes, Figure 4C). These later were used to calculate the mean size distribution on XY-planes, which was compared to that obtained with the 3D-segmentation data (Figure 4D). In the 2D analysis, the mean numbers of small (<200 μm) and large (>900 μm) follicles were lower, whereas the mean numbers of intermediate follicles were higher (dotted line), as compared to the 3D analysis (continuous line). In addition, 2D diameters <50 μm could be measured, although the 3D segmentation process was restrained to follicles above 50 μm in diameter. Altogether these data reveal the important bias introduced by the 2D analysis.

Three-dimensional follicular distribution in the medaka ovary

Three ovaries were processed as previously described through the MG staining and C-ECi clearing followed by 3D imaging, 3D segmentation and follicular diameter measurements. The mean size distribution of follicles, ranging from 56 to 1033 μm diameter, was obtained (Figure 4E). The developmental stage of follicles was determined according to their diameter and as described in the oocyte developmental table of Iwamatsu et al. [27]: 50–150 μm diameters (stage of previtellogenesis, stages II–IV, in yellow); 150–400 μm diameters (stage of pre-yolk formation, stages V and VI, in magenta), 400–500 μm diameters (early-yolk formation stage, stage VII, in green), 500–800 μm diameters (late-yolk formation stage, stage VIII, in light blue), and >800 μm diameters (maturating stage, stage IX, in dark blue). The mean follicular size distribution displays a predominance of small previtellogenic follicles (56.6%, in yellow) and pre-yolk formation follicles (29.4%, in magenta). Representative 3D views of follicles at the different stages indicated that the large maturing follicles (in dark blue) were preferentially located on the ventral side of the ovary, whereas no evident spatial arrangement could be observed for the other categories (Figure 4F).

C-ECi clearing, 3D imaging, and 3D segmentation of the trout ovary

The C-ECi clearing, 3D imaging, and 3D segmentation procedures were tested, as a proof of concept, on the rainbow trout ovary that has much larger ovaries and oocytes (up to 4–5 mm) than the medaka ovary. Pieces of trout ovary (about 8 × 6 × 2 mm) collected from an adult female fish were processed through the C-ECi protocol and MG staining. Opacity was sufficiently reduced to see through the samples (Figure 5A). A sample was used for 3D imaging by confocal microscopy (Figure 5B), and the whole volume was reconstructed in 3D (Figure 5C). No major loss of resolution was observed on XY-planes at increasing depths (Figure 5D, left panels and Supplementary Video S2). Follicles were segmented from the 3D image data using a computational semi-automatic 3D segmentation procedure. Few follicles could not be segmented (Figure 5D, right panels, arrows). The resulting 3D surface reconstruction of follicles...
Figure 5. 3D imaging and 3D analysis of a portion of trout ovary after C-ECi clearing. (A) A portion of a trout ovary before and after clearing with C-ECi and MG staining. (B) Schematic overview of the sample mounting for confocal imaging of trout ovary portions. (C) 3D reconstruction after C-ECi clearing, MG staining, and confocal imaging. (D) XY-planes at increasing depths showing image data (left panels) and follicular 3D segmentation data (right panels). Few follicles at
displayed different populations of growing follicles of various sizes (Figure 5E). The same correction factor as for the medaka follicles was applied to estimate the real follicular diameters of a total of 499 follicles (Figure 5F). The size distribution of follicles, ranging from 50 to 993 μm diameter, was obtained. The developmental stage of follicles was determined according to their diameter and as described in previous studies [30–32]: 50–250 μm diameters (early perinucleolar stage, in yellow), 250–450 μm (late perinucleolar stage, in magenta), 450–800 μm diameters (cortical alveoli stage, in light blue), and >800 μm diameters (peripheral yolk granule stage, in dark blue). The resulting profile displayed a large predominance of small early perinucleolar follicles (78.36%, in yellow), and no evident spatial arrangement was observed in the lamella structures on 3D views of each category of follicles in this sample (Figure 5G).

To conclude, using the C-ECi protocol, we were able to analyze the follicular size distribution in the ovary of both medaka and trout, which was till now limited to late-vitellogenic and postvitellogenic follicles [33]. To our knowledge, this is the first procedure elaborated for clearing and imaging fish ovary in 3D. The main limitation of this approach is the follicular segmentation from image data, the tissue shrinkage not being considered as a major drawback as discussed previously. The 3D segmentation of image stacks indeed failed to detect a small number of follicles. The main difficulty for 3D segmentation likely comes from a somehow discontinuous staining contrast in depth. One possibility would be to refine the segmentation algorithm that should greatly benefit from recent progress in machine learning approaches. Leading edge research in machine learning recently led to accurate algorithms and software libraries dedicated to image analysis, including image segmentation, and allowed the development of deep learning approaches for the detection and counting of follicles on 2D sections of mouse ovaries [34–36]. These recent progress are expected to contribute to the elaboration of a learning model dedicated to 3D follicular segmentation from 3D image data.

Anyhow, the C-ECi procedure is a nontoxic and efficient method for 3D follicular imaging that allows for the first time getting exhaustive and quantitative data on follicles enclosed at a given time in the fish ovary. We now expect that further kinetic analyses of the follicular content throughout the reproductive cycle of both medaka and trout will provide true quantitative data on the oogenesis dynamics in fish ovary. Beside the investigation of follicular contents, the analysis of the different cell lineages, including very early oocytes <50 μm in diameter (stage I), should bring complementary knowledge, such as the regulation of the early steps of oogenesis in the germinal cradle and the regulation of the granulosa cells differentiation. To this aim, we expect that the C-ECi clearing protocol, which combines both CUBIC and ECi methods, will be compatible with fluorescent cell markers other than MG, considering that both methods were previously individually conducted on various organs and species in combination with various fluorescent antibodies or fluorescent reporter genes [11, 13, 15, 20, 23, 37]. The next step will thus consist in combining the C-Eci protocol with fluorescent immunostainings or transgenic lines for further developmental and physiological analyses, which will open up new perspectives for a better understanding of the biology of fish ovary. We also expect that this method could be applied to other fish species, including zebrafish.

**Supplementary data**

Supplementary data is available at BIOLRE online.

**Acknowledgments**

We thank the INRAE-LPGP fish facility staff and especially Cecile Duret and Guillaume Gourmelen for fish rearing and husbandry.

**Conflict of interest**

No competing interests declared.

**Author contributions**

M.L. performed image processing, data analyses and participated in the manuscript writing. M.T. performed samples clarification, samples imaging, led the setup of the C-ECi protocol, and participated in the manuscript writing. J.Bu. performed the semi-automatic 3D segmentation workflow. A.B. participated in the setup of the clearing protocol. S.G. and E.C. collected samples. M.H. and F.M. participated in data analyses. J.B. participated in the design of the study. V.T. conceived the study, and participated in data analyses, and manuscript writing. All authors read and approved the final manuscript.

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Figure 5. (continued) the border are not segmented (arrows). (E) Top and bottom views of the 3D reconstruction of the whole sample merged with the 3D segmented follicles. (F) Distribution of 3D follicular diameters. The corresponding developmental stages are indicated with different colors (from early perinucleolar stage to peripheral yolk granule stage). The follicular size distribution displays a large predominance of small early perinucleolar follicles (in yellow). (G) 3D reconstructions of follicles at the different stages. No evident spatial arrangement of the different class of follicles is observed in lamella structures. Frequencies are percentages of the total number of segmented follicles ($n = 499$). Square $= 1 \times 1$ mm; Scale bars: 1 mm.
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