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Three-dimensional live imaging of bovine embryos by optical coherence tomography

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Abstract. While embryo transfer (ET) is widely practiced, many of the transferred embryos fail to develop in cattle. To establish a more effective method for selecting bovine embryos for ET, here we quantified morphological parameters of living embryos using three-dimensional (3D) images non-invasively captured by optical coherence tomography (OCT). Seven Japanese Black embryos produced by *in vitro* fertilization that had reached the expanded blastocyst stage after 7 days of culture were transferred after imaged by OCT. Twenty-two parameters, including thickness and volumes of the inner cell mass, trophectoderm, and zona pellucida, and volumes of blastocoel and whole embryo, were quantified from 3D images. Four of the seven recipients became pregnant. We suggest that these 22 parameters can be potentially employed to evaluate the quality of bovine embryos before ET.

Key words: 3D image, Blastocyst bovine embryo transfer, Embryo evaluation, Optical coherence tomography, Quantification of embryo structures

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Embryo transfer (ET) has been used widely to produce calves in combination with other reproductive technologies such as *in vitro* fertilization (IVF), nuclear transfer, and genomic breeding. The conception rate of ET in cattle is lower than that of artificial insemination, indicating the scope for improvement in ET technology. In particular, the conception rate of ET using IVF embryos is as low as 30–40%, which is inferior to that observed using embryos produced *in vivo* (around 50%) [1–4]. Furthermore, the transfer of IVF embryos has been reported to result in large offsprings with undesirably high birth weights, long pregnancies, and high miscarriage rates [5]. Embryos for transfer are usually selected based on observations using a conventional optical microscope at the time of transfer. The quality of the embryo is expressed by a code established by the International Embryo Technology Society (IETS) [6, 7], and highly skilled technicians are needed to perform this task.

In human artificial reproductive technology (ART), embryos are evaluated based on the Veeck and Gardner classification [8, 9]. Time-lapse cinematography (TLC) with a visible light microscope has recently emerged as a popular technology. In the evaluation of the *in vitro* developed bovine embryos, TLC has been used to determine the time of cleavage, number of blastomeres at first cleavage, and number of blastomeres at the onset of the lag phase [4, 10–12]. However, live bovine embryos have not been evaluated based on their three-dimensional (3D) structures.

Optical coherence tomography (OCT) is a non-invasive cross-sectional imaging process used in biological systems [13–15]. At present, it is used in ophthalmology, especially for funduscopic examination of the retina. Using stage-top modeled OCT, Takahashi *et al.* [15] reported a method for 3D live imaging of changes in the vessel structure during angiogenesis *in vitro*. Three-dimensional imaging using confocal microscopy is carried out for mouse embryos, which are exposed to weak ultraviolet light for observation [16]. As stage-top modeled OCT can be used to measure 3D images with high spatial resolution in living structures *in vitro*, we hypothesized that OCT can show 3D structures of living embryos, including their inside structures that could not be clearly observed by conventional optical microscopy. In the present study, to establish a new method for evaluation of bovine embryo for ET, we tried to obtain non-invasive, cross-sectional images of the external form and internal structure of the embryo using the OCT system and quantified several parameters based on the 3D images of bovine embryos. Furthermore, to confirm whether OCT scanning has cytotoxic effects on bovine embryos, we transferred OCT-scanned embryos to recipient cows and checked their fertility.

The OCT system scans the light source in the x-axis while shifting the scanning line positions on the y-axis to obtain a signal on the x-y plane at a focus position on the z-axis. We repeated this scanning while shifting z-axis focus positions to obtain 3D images of embryos

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(Fig. 1). The lengths of the imaging range on the x-axis, y-axis, and z-axis were 300, 300, and 200 µm, respectively. The exposure time was 150 µsec, and the scanning of an entire embryo could be completed in a few minutes. OCT provided cross-sectional images with a slice thickness of 1 µm, as shown in Fig. 1. OCT allows obtaining live images non-invasively without staining. It was possible to visualize the fine structure inside the embryo that could not be observed with an optical microscope (Fig. 2: representative images of an embryo [No. 2 in Table 1]). Before transfer, the embryo was imaged under a microscope (Fig. 2A) and by OCT. Based on the OCT images, trophectoderm (TE; cyan) and inner cell mass (ICM; magenta) were 3D-visualized (Fig. 2B). The structure of the whole embryo, including the ICM (red), TE (blue), and zona pellucida (ZP; gray), were also 3D-visualized (Fig. 2C). In addition, each part of the embryo, ICM (Fig. 2D), TE (Fig. 2E), and blastocoel (Fig. 2F), was individually 3D-visualized.

The values of the 22 parameters measured for the seven embryos are shown in Table 1. In embryos subjected to ET, the average thickness of ICM, TE, ZP, and TE + ZP were 50.9 ± 7.3, 3.8 ± 0.8, 14.3 ± 1.7, and 18.7 ± 1.2 µm, respectively. The average volumes of ICM, TE, ZP, TE + ZP, ICM + TE + ZP, blastocoel, and whole embryo were 3.2 ± 0.4, 2.0 ± 0.6, 15.0 ± 0.2, 17.4 ± 1.9, 20.6 ± 1.6, 12.8 ± 2.3, and 33.4 ± 3.7 × 10^5 µm^3, respectively. The blastocoel diameter was 51.7 ± 2.6 µm. Four of the seven recipients became pregnant.

The present results describe the first 3D imaging of bovine embryos using OCT. The images clearly revealed the internal structures of the embryos, otherwise difficult to observe under a microscope. The 3D images could also be used to calculate morphological parameters such as volume of the parts of the embryo (whole, blastocoel, ICM,

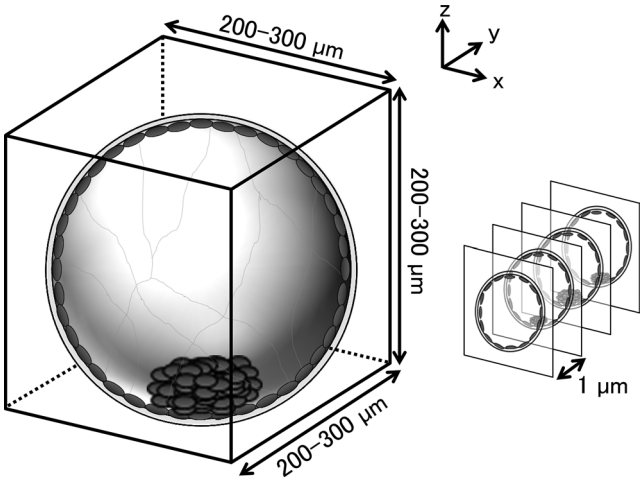

Fig. 1. Scanning scale for bovine embryo. Longitudinal imaging was performed in the area of bovine embryo.

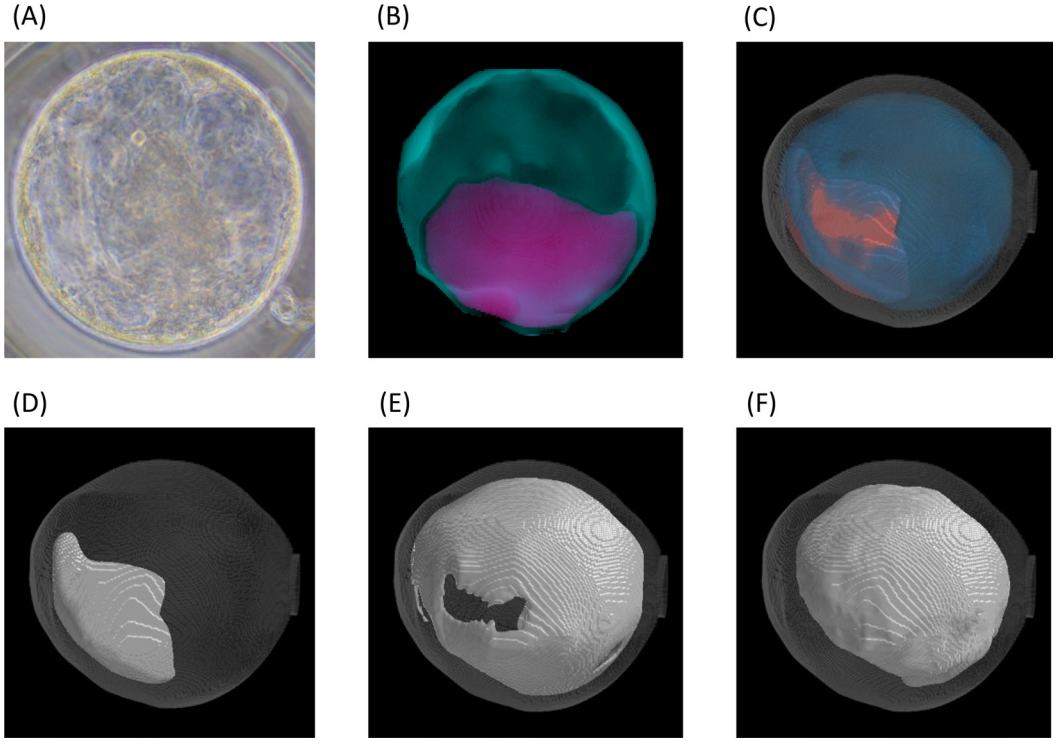

Fig. 2. Optical coherence tomography (OCT) images of a transferred bovine embryo (embryo No. 2 in Table 1). (A) Transferred embryo imaged by a microscope. This embryo was determined as Code 1 according to the IETS codes. (B) Sum of all pixel values in z-stack images of trophectoderm (TE; cyan) and inner cell mass (ICM; magenta) part was extracted from the tomographic image and synthesized 2D image. (C) 3D visualization of structures of an embryo, including ICM (red), TE (blue), zona pellucida (ZP; gray), and blastocoel. (D–F) 3D visualization of each structure of an embryo: ICM (D), TE (E), and blastocoel (F).

Table 1. Quantification of 22 parameters in bovine embryo (n = 7)

		Embryo No.							
		1	2	3	4	5	6	7	mean ± SD
Structual thickness (μm)									
ICM	Mean	38.1	56.2	42.2	53.2	51.8	59.9	55.2	50.9 ± 7.3
	Median	38.0	57.1	43.1	55.0	55.1	61.3	56.2	52.3 ± 7.8
	SD	5.9	8.2	5.5	7.4	9.1	8.5	7.1	7.4 ± 1.3
TE	Mean	3.1	3.7	3.0	4.3	5.0	4.7	3.0	3.8 ± 0.8
	Median	2.5	2.6	2.4	3.1	3.2	3.6	2.5	2.9 ± 0.4
	SD	2.4	3.4	2.6	4.1	5.1	4.3	3.1	3.6 ± 0.9
ZP	Mean	16.4	14.9	16.6	13.3	11.4	13.8	13.6	14.3 ± 1.7
	Median	17.0	15.4	17.1	13.4	12.0	15.0	14.3	14.9 ± 1.7
	SD	2.5	2.1	2.6	1.9	1.8	3.0	3.0	2.4 ± 0.5
TE + ZP	Mean	19.7	19.4	19.6	17.7	16.7	19.8	17.9	18.7 ± 1.2
	Median	19.9	19.0	19.5	17.0	15.1	19.1	18.0	18.2 ± 1.6
	SD	3.9	3.8	4.0	4.8	5.3	4.9	4.1	4.4 ± 0.6
Volume ($\times 10^5$ μm^3)									
	ICM	2.5	3.1	2.7	3.6	3.7	3.3	3.6	3.2 ± 0.4
	TE	1.2	2.4	1.4	1.8	2.6	2.7	1.7	2.0 ± 0.6
	ZP	16.2	14.4	20.0	13.7	11.9	14.8	13.9	15.0 ± 0.2
	TE + ZP	17.5	17.4	21.4	16.0	15.5	18.1	16.0	17.4 ± 1.9
	ICM + TE + ZP	20.0	20.5	24.1	19.6	19.2	21.5	19.5	20.6 ± 1.6
	Blastocoel	11.4	11.3	18.1	12.0	13.8	11.5	11.2	12.8 ± 2.3
	Whole embryo	31.4	31.9	42.2	31.5	33.0	33.0	30.7	33.4 ± 3.7
Diameter of blastocoel (μm)									
	Mean	49.3	50.7	57.6	50.7	53.2	50.3	50.4	51.7 ± 2.6
	Median	51.6	52.7	60.3	52.9	55.7	52.5	52.4	54.0 ± 2.8
	SD	13.3	14.5	15.6	14.1	14.8	14.1	14.5	14.4 ± 0.7
ET status									
	IETS Code	Code 1	Code 1	Code 1	Code 1	Code 1	Code 1	Code 1	
	Pregnancy	–	+	+	+	–	+	–	

ICM, inner cell mass; TE, trophectoderm; ZP, zona pellucida.

and TE). These parameters may be useful for evaluating embryo quality before ET. In human ART, several parameters such as embryo development stage, blastocoel volume, ICM, and TE thickness are recognized as assessment criteria. The operator subjectively determines the scores of these parameters by microscopic observation [17, 18]. The present study shows that a stage-top OCT system could serve as a new way of objectively evaluating the quality of bovine embryos. Bovine embryos for ET are usually evaluated by the IETS codes [6, 7] whose accuracy relies on the proficiency of the operators. Sugimura *et al.* [4, 11] showed that high-quality bovine embryos could be selected using morphokinetic indicators such as timing, number of blastomeres at first cleavage, and number of blastomeres at the onset of the lag phase as well as by observing nuclear/chromosomal abnormalities of bovine embryos using live-cell imaging technology [19]. Thus, morphological indices contribute to the critical selection of high-quality bovine embryos. Therefore, OCT images that allow observation of morphological details of the internal structure may also be useful to evaluate the quality of bovine embryos. At 168 h post-insemination (hpi), embryos that were classified as IETS Code 1 were used for imaging. As bovine embryos contain much more lipids than human or mouse embryos, pronucleus formation in early embryos could not be confirmed under a microscope, posing difficulty in evaluation of their quality [20]. Zheng *et al.* [21] used OCT to observe the 3D morphology of mouse early embryos (mono-, 2-, and 4-cell stages). These authors succeeded in label-free visualization of the male and female pronucleus before the first cleavage and the nucleus in the blastomere after cleavage [21]. In cattle, OCT imaging of early embryos may be useful for visualizing pronuclei and detecting morphological abnormalities that are difficult to observe using a conventional microscope. The OCT system used in the present study has been suggested to provide effective information for the evaluation of bovine embryos by the precise observation of the 3D structure of the embryo. The present results show OCT images only in the embryos classified as Code 1 of IETS codes. Therefore, OCT evaluation of embryos in other IETS codes is warranted to confirm its applicability for the morphological evaluation of bovine embryos.

Using the OCT system, we could quantify the internal structure of bovine embryos. IETS codes grade embryos according to their developmental stages and the status of degeneration of the cells in

the embryo but provide no information about the volume of the whole embryo, ICM, or TE [6]. In human ART, Gardner classification is insufficient for morphological selection of good embryos; however, morphological parameters of TE have been suggested to be more closely associated with live birth than those of ICM [22]. As morphological parameters of TE were also quantified by OCT in the present study, OCT may support the selection of good bovine embryos for transfer.

The growth potential of early embryos relies on the total cell number of blastocysts, especially the composition ratio of ICM and TE [23]. The number of cells in a bovine embryo is usually counted by double staining with Hoechst 33258 and propidium iodide [23]. However, stained embryos cannot be used for ET. The OCT system uses near-infrared light (λ: 900 nm), which has low cytotoxicity [24–26], and allows non-invasive observations of bovine embryo morphology; therefore, OCT-scanned embryos can be used for ET. We have also used the same OCT system to observe mouse embryos before ET and achieved a normal delivery after ET (Ohbayashi *et al*., unpublished data). In the present study, we achieved conceptions after transferring bovine embryos imaged by the same OCT system used in mice. A few minutes of OCT did not change the embryo morphology. Together, these findings indicate that using the OCT system could allow visualization of the internal 3D structure and quantification of morphological parameters in living bovine embryos. This new method has the potential to support the objective evaluation of bovine embryos before ET. The comparison of the values of each parameter found in the present study between pregnancy and non-pregnancy is important to determine whether the OCT system is available for embryo evaluation for ET.

Methods

Ethics statement

Animal handling and experimental procedures were carried out following the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan (http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf).

Experimental design

Bovine IVF embryos were derived from oocytes obtained by ovum pick-up (OPU) and cryopreserved until the time of ET. We investigated their morphological structures by OCT and determined their fertility following transfer into recipient cattle.

Production of embryos derived from oocytes collected by OPU and in vitro maturation (IVM)

As previously described [27], cumulus-oocyte complexes (COCs) were collected from a 73-month-old Japanese Black cow using an ultrasound scanner (HS-2100; Honda Electronics, Toyohashi, Japan) and a 7.5-MHz convex array transducer (HCV-4710MV; Honda Electronics) with a 17-gauge stainless steel needle guide. Follicles > 2 mm in diameter were aspirated with the vacuum through a disposable aspiration needle (COVA Needle; Misawa Medical, Tokyo, Japan). The aspiration rate was 14 ml/min, and the vacuum pressure was 100 mmHg. The IVM medium was 25 mM HEPES-buffered TCM199 (M199; Gibco, Paisley, Scotland, UK), supplemented with 10% newborn calf serum (NCS; 16010159, Gibco) and 0.01 AU/ml follicle-stimulating hormone from porcine pituitary (Antorin-R10; Kyoritsu Seiyaku, Tokyo, Japan). COCs with two or more granulosa layers were washed thrice with IVM medium, and the recovered COCs were cultured in four-well dishes (Non-Treated Multidishes; Nalge Nunc International, Roskilde, Denmark) in 600 µl of IVM medium. The cultures were covered with mineral oil (M8414; Sigma-Aldrich, St. Louis, MO, USA) and incubated for 21–22 h at 38.5°C in 5% CO_2, 5% O_2, and 90% N_2 with humidified air. All cultures were maintained under these conditions.

IVF

Frozen semen from Japanese Black bulls stored in straws was thawed in water at 37°C for 40 sec. After centrifugation (840 × *g*, 5 min), the supernatant was removed and the sperm suspension with a final sperm concentration of 1.0×10^7/ml diluted by IVF100 (Research Institute for the Functional Peptides, Yamagata, Japan) served as the IVF medium. After 22 h of IVM, the COCs were removed from the IVM medium and washed twice with IVF100. Up to 20 COCs were incubated in 35 mm dishes (Falcon 351008; Corning, NY, USA) containing 100 µl droplets of IVF medium for 6 h.

In vitro culture (IVC)

After insemination, oocytes were completely denuded from cumulus cells and spermatozoa by repeated pipetting with a glass pipette in the IVC medium, potassium simplex optimized medium (KSOM) with amino acid (KSOMaa Evolve Bovine; Zenith Biotech, Bangkok, Thailand) supplemented with 5% NCS and 0.6 mg/ml L-Carnitine (C0158, Sigma-Aldrich). Presumptive zygotes were subsequently washed thrice with IVC medium and cultured for 48 h in 100 µl droplets of IVC medium. Each droplet contained approximately 20 presumptive zygotes. At 48 hpi, embryos with more than four cells were transferred from 35 mm dishes to well-of-the-well (WOW) dishes (LinKID micro25, Dai Nippon Printing Co., Ltd., Tokyo, Japan) as previously described [10]. WOW dishes, which are 35 mm in diameter, have 25 microwells (5 columns × 5 rows) and a circular wall in the center. A WOW dish can culture up to 25 embryos each with a single drop of medium, and allows tracking of individual embryos. Pre-cultured IVC medium (100 µl) was placed within the circular wall and covered with mineral oil. At 168 to 180 hpi, embryos that had developed to or beyond the blastocyst stage were observed under an inverted microscope.

OCT observations

IVF embryos were cultured for 7 days (such that they had reached the expanded blastocyst stage) and examined under an inverted microscope. Only embryos that were independently classified as IETS Code 1 by three skilled observers were used. OCT imaging was performed as previously described [15].

Unstained live embryos were imaged by OCT using a Cell3iMager Estier (SCREEN Holdings Co., Ltd., Kyoto, Japan). Values for multiple parameters based on the Gardner classification were obtained from the 3D image data. The system is equipped with a superluminescent diode (SLD; center wavelength: 890 nm, N.A. = 0.3). The SLD output is coupled to a single-mode optical fiber and split at an optical fiber coupler into the sample and reference arms. The reflections from the

two arms were combined at the coupler and detected by a spectrometer. The 3D image data of the embryos were constructed from individual 2D x–z cross-sectional images, which were obtained by a series of longitudinal scans by laterally translating the optical beam position. The data acquisition window was 200–300 × 200–300 × 200–300 µm, and the voxel size was 1 × 1 × 1 µm (Fig. 1).

Cryopreservation

As described previously [28], blastocysts imaged by OCT were transferred to a cryoprotective solution (1.8 M ethylene glycol and 0.1 M sucrose in Dulbecco's phosphate buffered saline [D-PBS]), which was then placed in a 0.25 ml straw (IMV Technologies, L'Aigle, France) at room temperature (25°C). After the blastocysts were equilibrated at room temperature for 15 min, the straws were directly set in a programmable freezer (ET-1N; FUJIHIRA INDUSTRY CO., LTD., Tokyo, Japan) at –7°C where seeding was manually performed. The straws were subsequently cooled at a rate of 0.3°C/min to –30°C and then directly transferred to liquid nitrogen for storage until use.

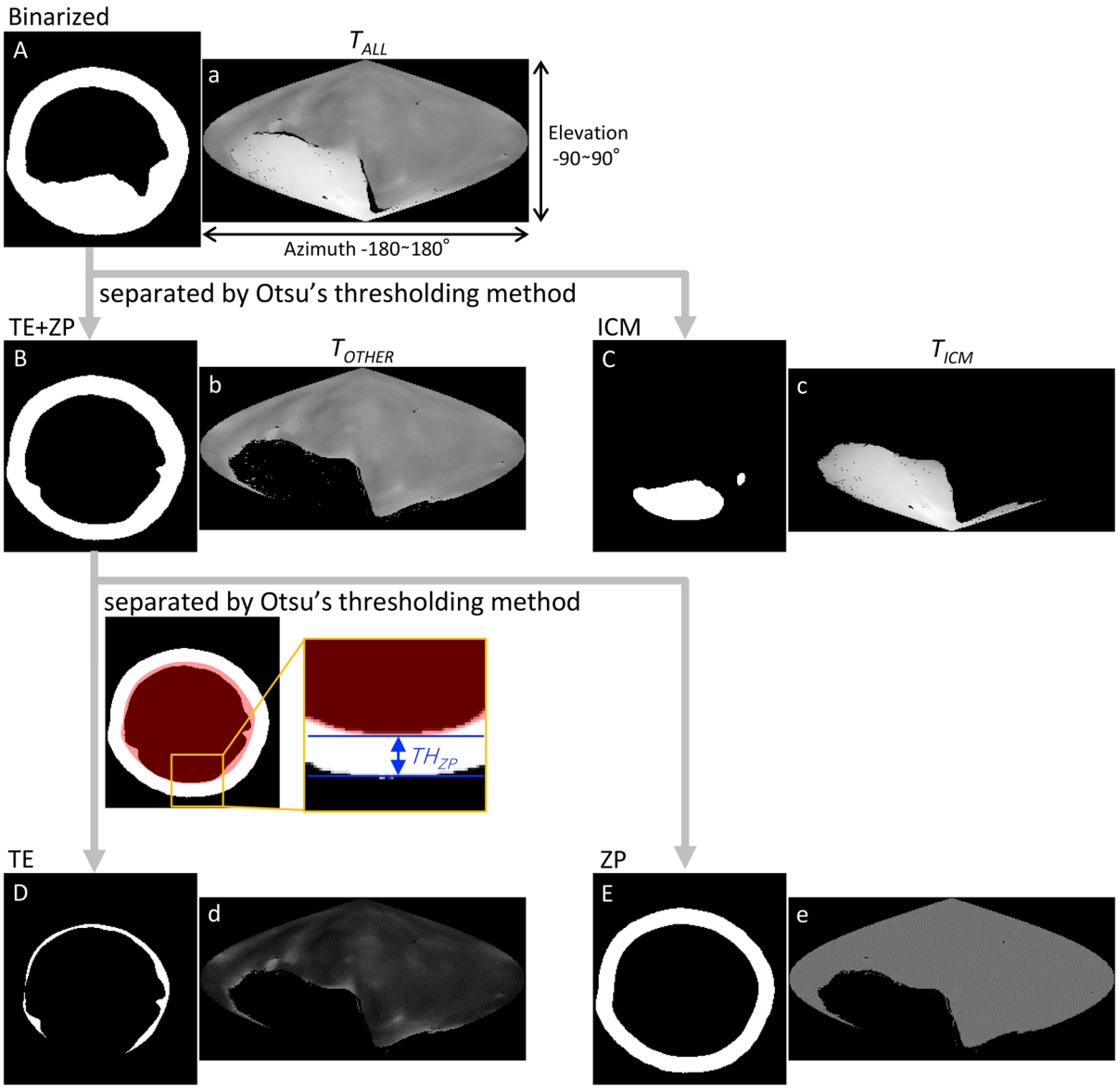

Fig. 3. 3D image analysis of bovine embryos. The binarized image was separated into inner cell mass (ICM), trophectoderm (TE), and zona pellucida (ZP). (Panels A & a) Thickness of embryo (T_{ALL}) was measured by drawing vectors from center to outer surface and inner surface (the outermost of blastocoel) of the embryo. ICM parts (Panels C & c) were extracted from T_{ALL} by the Otsu's thresholding method [29, 30]. (Panels B & b) The parts that remained after removing ICM parts from T_{ALL} were defined as T_{OTHER}. T_{OTHER} were separated using the Otsu's thresholding method into TE (Panels D & d) and ZP (Panels E & e) by calculating their average thickness [29, 30]. A–E: A 2D image derived from a binarized 3D image. a–e: Sinusoidal projections for each part of a 3D image. TH_{ZP}: Threshold of ZP.

The straws were thawed in air for 10 sec and then immersed in a water bath at 30°C for 20 sec for ET.

Image analysis

The image analysis process is briefly shown in Fig. 3. The 3D images of bovine embryos were binarized. The binarized image was separated into ICM, TE, and ZP. The thickness of the embryo (T_{ALL}) was measured by drawing vectors from the center to the outer surface and inner surface (the outermost of blastocoel) of the embryo in the elevation direction (–90 to 90 degrees) and the azimuth direction (–180° to 180°). ICM parts were extracted from T_{ALL} by Otsu's thresholding method [29, 30]. The parts that remained after removing ICM parts from T_{ALL} were defined as T_{OTHER}. T_{OTHER} were separated using the Otsu's thresholding method into TE and ZP by calculating their average thickness [29, 30].

ET and pregnancy diagnosis

The OCT-imaged embryos were transferred to seven 3.9 ± 1.2-year-old recipient Holstein cows in February and March 2019 at a commercial farm in Tottori prefecture, Japan. The cows were clinically normal with body condition scores (BSC) between 2.75 and 3.0. The BCS scale ranges from 1 to 5 with 0.25 increments. Before ET, recipients were estrus-synchronized by administration of a CIDR device (CIDR 1900; Zoetis Japan, Tokyo, Japan) for 9 days and treated with cloprostenol (Dalmazin 150 µg im; Kyoritsu Seiyaku Corporation, Tokyo, Japan) 2 days before CIDR removal. The estrus of recipient cows was monitored, and embryos were transferred 7 days after estrus. The recipients were examined for pregnancy 23 days after ET using ultrasonography (HS101V; Honda Electronics). Pregnancy was confirmed by observation of the embryonic membrane and the embryo with a detectable heart beat in the intraluminal uterine fluid.

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