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Optical tissue clearing in combination with perfusion and immunofluorescence for placental vascular imaging

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Abstract

Imaging of placental tissues is a difficult task, because of specific for this organ complex multicellular and 3D tissue structure. The tissue clearing systems (X-CLARITY) system is a valuable tool for the examining the expression of molecular pathways in whole tissues and organs, originally developed for brain imaging.

In the present report, we utilized this technology for the examination of placental vasculature and protein expression in perfused human placental tissue.

The placental tissue was sufficiently cleared with preservation of endothelial staining and fluorescent markers, allowing visualization using confocal microscopy. The CLARITY method and X-CLARITY system is a valuable tool in placental imaging.

Abbreviations: BODIPY-C12 = 4,4-difluoro-5-methyl-4-bora-3a, 4a-diaza-s-indacene-3-dodecanoic acid, 3D = 3-dimensional, DiI = 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocaninperchlorate, FITC = fluorescein isothiocyanate, PBS = phosphate-buffered saline, X-CLARITY = tissue clearing systems.

Keywords: 3D, image clearing, perfusion, placenta, vasculature

1. Introduction

The placenta is poorly understood, but is one of the most important organs of the body,[1] since it supports fetal growth. In turn, fetal growth is closely related to the vascular architecture of placenta.[2] Pathology of placental vascular development is associated with abnormal fetal development.[3,4] The preservation of villous architecture is essential for evaluation of vascular composition since maternal and fetal pressure are factors, which estimate placental vascular-syntial membrane morphology.[5] The complex placental structure represents significant burden in evaluation of placental vascular tree.[6] The method of "clearing" a whole mouse brain by transforming it into a porous hydrogel-hybridized form that retains tissue characteristics such as receptor location and other natural structures was originally developed by Deisseroth group[7] and was applied to human placenta previously.[8] The tissue clearing systems (X-CLARITY, Logos Biosystems, VA) is capable of clearing tissue in half the time of the original method.[7,9–11]

Here, we applied this method to perfused human placental tissue after vascular staining, allowing for the examination of the intact vascular architecture and evaluation of the spatial expression of lipids and proteins.

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2. Materials and methods

2.1. Placental collection and general protocol

Four placentas were collected within 1 to 2 minutes after delivery (this study was approved by TTUHSC Institutional Review Board, protocol #L15–108) and an isolated placental cotyledon was cannulated within 5 to 7 minutes after collection. Placental perfusion was performed in accordance with the *ex vivo* placental perfusion protocol.[6] Details of the tissue processing are presented in Table 1.

2.2. Fluorescent markers used in placental perfusion

The fetal and maternal perfusion closed systems were established and perfusion was performed for at least 90 minutes. 4,4-Difluoro-5-methyl-4-bora-3a, 4a-diaza-s-indacene-3-dodecanoic acid (BODIPY-C12, Cat. No. D3823, ThermoFisher Scientific; Waltham, MA) was added to the maternal side at the concentration 2 μM according to the previously described protocol.[12] Fluorescein isothiocyanate (FITC)-conjugated dextran (Cat No. FD70S, Sigma–Aldrich, St Louis, MO) was added to fetal buffer at the working concentration of 200 μg/mL, perfusion was performed during 90 to 120 minutes.[13]

Table 1

Details of experimental procedures.

Placental ID	Perfusion protocol/fetal flow rate	Endothelial staining DiI	BODIPY-12	FITC – conjugated Dextran in fetal circuit	Time in the fixative, d	Tissue embedding	Immunohistochemistry after CLARITY treatment	Images (presented on Fig. 1)
1	High fetal flow rate (12 mL/min)	Yes	No	No	20	4% Agarose	No	D
2	Initial perfusion	Yes	No	No	At least 7 days	4% Agarose	No	F
3	Fatty acid transfer (6 mL/min)	Yes	Yes	No	1	4% Agarose	Yes	E, G–J
4	High fetal flow rate (12 mL/min)	Yes	No	Yes	7	X-CLARITY mounting medium	Yes	K, L

BODIPY-C12 = 4,4-difluoro-5-methyl-4-bora-3a, 4a-diaza-s-indacene-3-dodecanoic acid, DiI = 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocaninperchlorate, FITC = fluorescein isothiocyanate, X-CLARITY = tissue clearing systems.

2.3. Endothelial staining

At the end of perfusion, each cotyledon was infused with 12 mL of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocaninperchlorate (DiI, Cat No. 42364, Sigma–Aldrich; St. Louis, MO) at the rate 6 mL/minutes, and subsequently with 12 mL of 4% paraformaldehyde at the same flow rate, using modified published protocol.[14] Subsequently a portion of the cotyledon was fixed for 1 to 7 days in 4% paraformaldehyde (4.0 °C).

2.4. Tissue embedding

All but one of the placental specimens were embedded in 4% agarose and sections with 300 to 400 μm thickness were cut with vibratome (The Vibratome Co, St. Louis, MO).

2.5. Tissue clearing using the X-CLARITY system

The tissue was removed from the fixative, rinsed with 1× phosphate-buffered saline (PBS) several times and then incubated in 1× PBS at 4.0°C for 24 hours. Afterward the tissue was immersed in the X-CLARITY polymerization initiator solution and processed according to manufacturer's instructions. Briefly, the samples were incubated for 24 hours at 4.0°C, polymerized for 3 hours at a vacuum (kPa) of −85, and a temperature of 37.0°C, shaken for 1 minute and rinsed 1× PBS. Subsequently samples were processed in the electrophoretic tissue clearing (ETC) solution for 20 hours (checked after 2 hours) at a current of 1.1 A, temperature of 37.0°C and pump speed of 30 rpm. Tissue was stored at 4.0 °C for 1 week. The primary antibody Perilipin 2 (Cat No. ab181452, Abcam; New York, NY) was prepared at a dilution of 1:100 in antibody dilution solution (1× PBS, 6% bovine serum albumin, 0.1% Triton X-100 and 0.01% sodium azide) and incubated at 37.0°C with specimens for 5 days. The secondary antibody, Alexa 488 (Cat No. A-11029, ThermoFisher; Austin, TX), was prepared at a 1:100 dilution in antibody dilution solution (same as above) and the tissue was incubated in this antibody solution for an additional 5 days. Subsequently, samples were stored in 1× PBS at 4.0°C. Before taking images, the samples were placed in X-CLARITY mounting solution for 1 hour. All fluorescent images were taken using the T1-E microscope with A1 confocal and STochastic Optical Reconstruction Microscopy super-resolution modules (Nikon Instruments Melville, NY).

2.6. Image processing

Images were processed using Imaris, 9.0.1 software (Bitplane, Oxford Instruments, concord, MA) and Image-Pro Premier 9.1 software (Hamamatsu, Japan).

3. Results

The placental tissue was sufficiently cleared and the refractive index (RI) matched well with the X-CLARITY mounting solution, providing maximal clarity (Fig. 1A–C). Fetal vascular tree was visualized after cleaning of the specimen with the preservation of the endothelial staining (Fig. 1H), when compared to the conventional method (Fig. 1D–F) (see video 1, Supplemental Video, http://links.lww.com/MD/C521 [Video 1: DiI staining (red), conventional method]). The endothelial staining was fading, when specimen was kept in fixative for 7 days prior to clearing (Fig. 1F (see video 2, Supplemental Video, http://links.lww.com/MD/C522) [Video 2: DiI staining (red), overlay image with auto fluorescence (green), conventional method, 7 days fixation]), L (see video 3, Supplemental Video, http://links.lww.com/MD/C523) [Video 3: Placental tissue perfused with DiI (red) and FITC-conjugated dextran, tissue clearing, 7 days fixation]). The BODIPY-C12 was localized on syncytiotrophoblast (ST) and endothelial cells (Fig. 1E), image clearing resulted in absence of ST staining (Fig. 1G–J) (see video 4, Supplemental Video, http://links.lww.com/MD/C524 [Video 4: Overlay of DiI (red) and perilipin/ BODIPY-C12 staining (green), tissue cleaning]). Subsequent to clearing, staining with perilipin-2 demonstrated peri- and endovascular localization of the lipid-related molecules (Fig. 1I (see video 4, Supplemental Video, http://links.lww.com/MD/C524 [Video 4: Overlay of DiI (red) and perilipin/ BODIPY-C12 staining (green), tissue cleaning]), J). Perfusion of fetal side with FITC-conjugated dextran enhanced green fluorescence background staining (Fig. 1K (see video 5, Supplemental Video, http://links.lww.com/MD/C525 [Video 5: FITC-conjugated dextran staining after fetal perfusion (green fluorescence), tissue clearing]), L (see video 3, Supplemental Video, http://links.lww.com/MD/C523 [Video 3: Placental tissue perfused with DiI (red) and FITC-conjugated dextran, tissue clearing, 7 days fixation])). In the later feto-maternal buffer transfer was above acceptable 3 mL/hour, when fetal flow rate was increased to 12 mL/minute.

4. Discussion

The ability to examine intact placental vasculature in the human placenta is a difficult task, hampered by the complex structure of this organ.[15] Many methods for characterization and quantification of placental vasculature have been published including vascular casting,[16,17] and 3-dimensional (3D) computational modeling.[18] However, thus far, none of the methods have been able to provide an undisturbed visualization of the placental vasculature and expression of functional lipids and proteins in placental endothelial cells in 3D images. Tissue clearing has been used for visualization of placenta in rodents[11] and for the

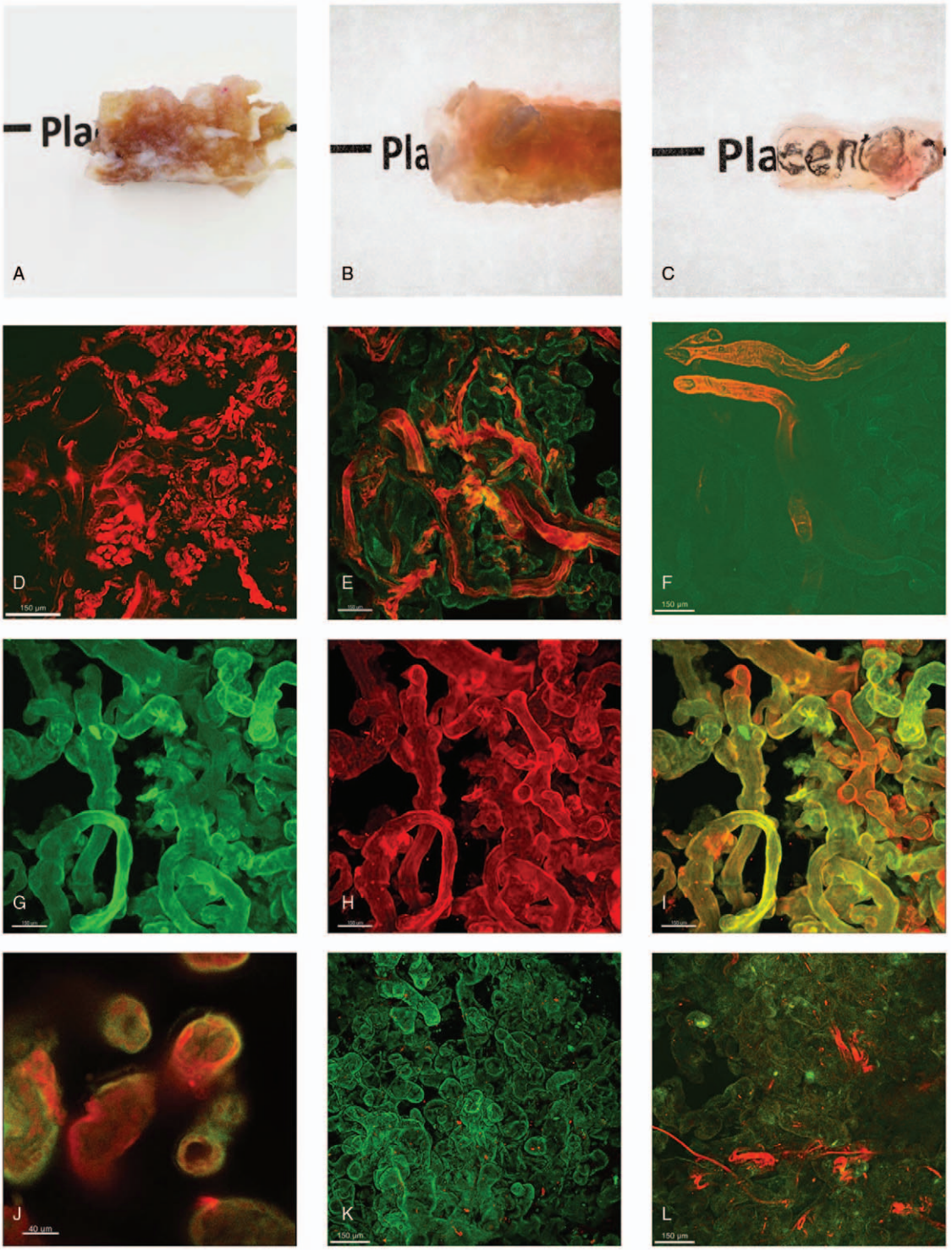

Figure 1. Imaging of placental tissue. Upper panel: (A–C) tissues before and after clearing with CLARITY system. Upper panel: (A) tissue before clearing; (B) tissue after clearing, before placing in mounting medium; and (C) cleared tissue placed in mounting medium. Second panel: (D–F) tissues were not cleared. (D) DiI staining, (E) BODIPY-C12 perfusion and DiI staining, and (F) DiI staining, overlay image with auto fluorescence. Third panel: (G–I)tissue cleared with CLARITY system. (G) Green fluorescence: BODIPY-C12 perfusion and Perilipin-2 staining. (H) Red fluorescence: DiI staining, (I) overlay of DiI and perilipin/BODIPY-C12 staining. Low panel: (J) overlay of DiI and perilipin/BODIPY-C12 staining, tissue cleaning; (K) green fluorescence: Perilipin-2 staining in the placental tissue perfused with DiI and FITC dextran, (L) controls (omission of secondary antibody green fluorescence: Perilipin-2 staining in the placental tissue perfused with DiI and FITC dextran). Notice: In all images: red fluorescence is DiI (vascular endothelial staining) and green fluorescence, is BODIPY-C12, Cat. No. D3823, ThermoFisher Scientific; Waltham, MA), perilipin-2 after tissue clearing or FITC conjugated dextran. Image A was taken using Nikon D3200, AF-S Nikon 18 to 55 mm lens, image C using a Canon 5D Mark II with a 100 mm macrolens camera on a copy stand with trans-illumination. Images D–I and K,L were taken with a 10× objective on a Nikon T1-E microscope with A1 confocal and STORM super-resolution modules. Images E, J, H, I, and J were adjusted using Imaris, 9.0.1 software; images D, F, K, and L were adjusted using Image-Pro Premier 9.1 software. Tissue in D–J were mounted in agarose gel and sliced to 300 µm with vibratome, K, L whole tissue in CLARITY mounting medium in a petri dish each ~5 mm. Scale bars 150 µm in all images except J with scale bar 40 µm. BODIPY-C12 = 4,4-difluoro-5-methyl-4-bora-3a, 4a-diaza-s-indacene-3-dodecanoic acid, DiI = 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocaninperchlorate, FITC = fluorescein isothiocyanate, X-CLARITY = tissue clearing systems.

evaluation of 3D structure of fetal membranes[19]/placental tissue in humans.[8] The novelty of the presented method is that the endothelial staining was performed prior to tissue clearing under physiological conditions (controlled temperature, flow rate), thus preserving the vascular architecture. By additional perfusion with BODIPY-C12 and FITC-conjugated Dextran prior to clearing and perilipin-2 immuno-staining after clearing, we were able to stain the placental vasculature as well as localize areas of lipid metabolism. Cleared tissue may also reduce the amount of "manual cleaning" needed in computational modeling when interpreting images.[18] The limitation of the technique at the present stage of development is the absence of the visualization of intravillous capillary loops, which are detectable with the other techniques.[20] Nevertheless, the application of this technology allows precise evaluation of 3D functional morphology of placental blood barrier and its transport capacities, which is an important question in the light of the development of nano-technologies and understanding of role of exosomes in fetal development.[21,22]

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