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Technical note

Clarification and 3-D visualization of immunolabeled human placenta villi

George Merz [a], Valerie Schwenk [a], Ruchit G. Shah [b], Phillip Necaise [a, b], Carolyn M. Salafia [a, b, *]

[a] New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314, United States
[b] Placental Analytics, New Rochelle, NY 10804, United States

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ABSTRACT

We report here the successful 3D visualization of human placenta villous structures on the order of ~1 mm^3 by a combination of immunolabeling, rapid tissue clarification and laser scanning confocal microscopy. The resultant image sets exhibit a complex arrangement of villi and their contained vasculature that mirrors their arrangement *in situ*.

1. Introduction

Villous trees are the conduits moving fetal blood from the chorionic plate to the distal terminal villi where transfer of nutrients, gases and waste occurs between the fetal and the maternal blood. Maternal conditions, such as pre-eclampsia, diabetes, maternal obesity, have lasting consequences for the development and distribution of these villous tree networks and their contained blood vessels [1–3] that effect not only the placenta and the developing fetus, but also the delivered infant, and the emergent adult [4–6].

Although confocal microscopy has been used to determine differences in the distribution of villous capillaries in normal and diabetic placentas [7] and to carry out computational modeling of the villous capillary and volume geometries [8], these studies were limited to examination of individual villi. Imaging and quantitative characterization of the network properties (branch points, branches, endpoints, loops, etc.) of a larger tissue volume (like on the order of a mm^3) is likely to give a more representative sample of placental tissue rather than stereological studies of individual villi.

Currently, no imaging mode provides a suitable approach to this scale. Scanning systems can readily capture the large volumes but cannot resolve "micro"-details. Microscopy, particularly confocal microscopy, has been limited by the scattering of light by refractive index mismatches of the tissue components such that useful imaging is limited to a depth of approximately 100 μm. In recent years, a number of techniques have emerged that get around this limit by correcting the refractive index mismatch, clarifying the tissue and thus allowing imaging to much greater depths [9]. We have used a new clarification technique, Visikol® HISTO™ [10], to clear samples of placenta tissue at 8, 12, and 18 weeks gestational age and term. These clarified tissues were immunolabeled and imaged with confocal microscope to demonstrate the efficacy of Visikol® HISTO™ in greatly improving the imaging resolution.

2. Methods

De-identified tissue samples were taken randomly from a tissue bank containing specimens from 120 elective pregnancy terminations between 7 weeks and 23 weeks, and delivered placentas. Villous fragments were dissected free of the chorionic plate and the basal plate; anchoring villi were excluded. Specimens were immediately fixed in 10% neutral buffered formaldehyde. Fig. 1 demonstrates the steps involved in isolation and immunolabeling of villous sample from a whole placenta.

* Corresponding author. New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314, United States.
E-mail address: carolyn.salafia@gmail.com (C.M. Salafia).

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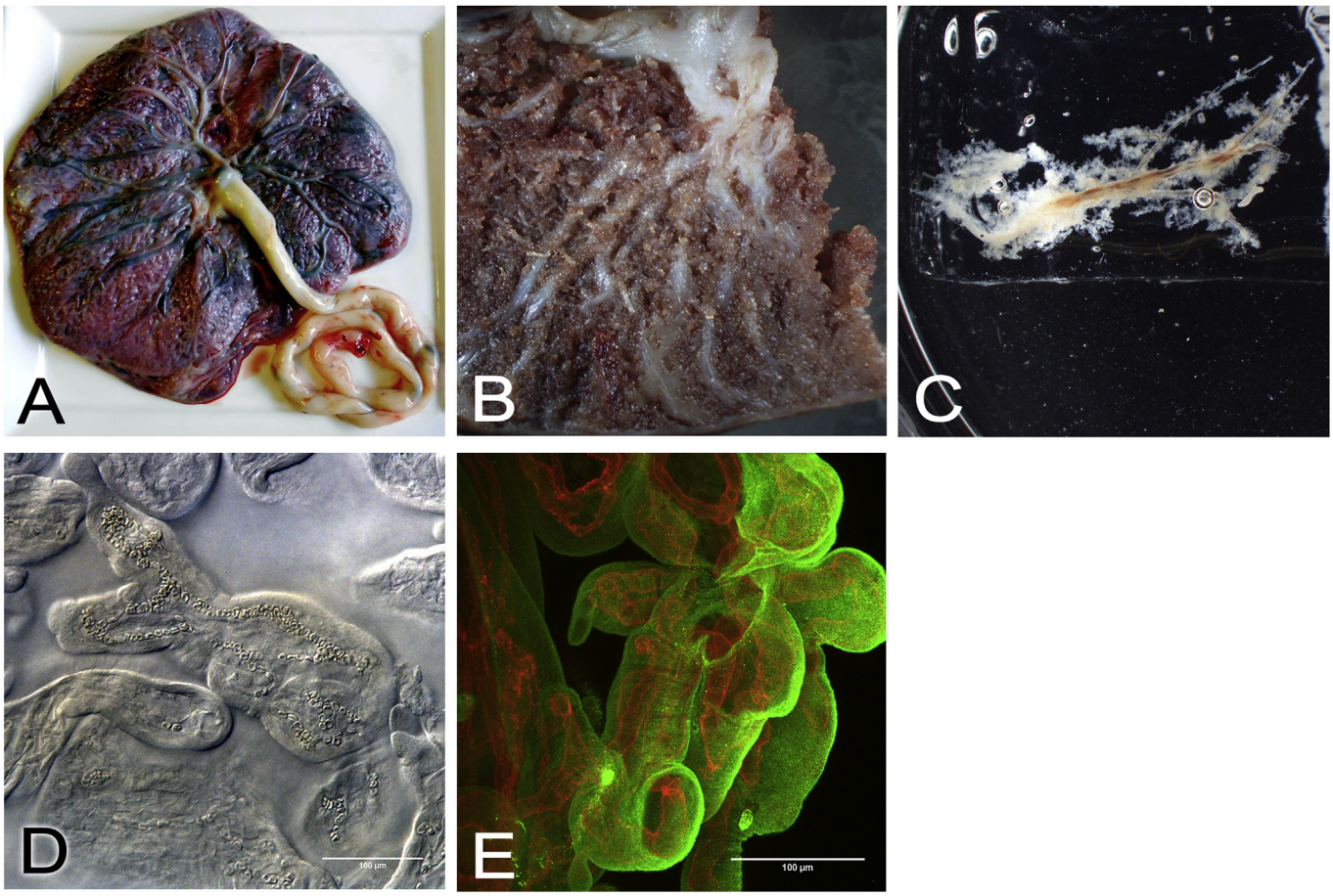

Fig. 1. Steps for isolation of villous sample from a whole placenta. A) whole placenta, B) cut face of placenta showing white large penetrating stem villi, C) ~1.5 cm segment of isolated villous tree, D) micrograph of a few terminal villi and capillaries (20x), E) confocal image of immunolabeled (CK7 green, CD31 red) terminal villi (40x). The scale bars in figures D) and E) are 100 μm each.

Dissected villous pieces were washed with phosphate buffered saline (PBS), permeabilized and blocked with PBS containing 0.1% triton-x100 + 2% goat serum for 1 h. The trophoblast layer and endothelial cells were immunolabeled with *anti*-CK7 and anti-CD31 (Thermo) in PBS+ 2% goat serum (PBS-GS). Pieces were incubated overnight at 4° C, and washed 3x with PBS-GS and incubated for 30 min in goat-*anti*-mouse IgG coupled with Alexa488 and goat anti-rabbit IgG coupled with Alexa633 (Invitrogen). For clarification, immunolabeled pieces were treated with the Visikol® HISTO™ protocol, i.e., dehydrated with three decreasing concentrations of ethanol (3 × 15′ for each of 50, 70,100%), followed by immersion in Visikol® HISTO™ Step 1 for 4 h, and finally transferred to Visikol® HISTO™ Step 2 for overnight incubation at room temperature.

Z- Stacks of the clarified tissues were acquired with a Nikon C1 laser scanning confocal system coupled to a Nikon 90i microscope with a 10x objective. The acquired, image stacks were processed by blind deconvolution (AutoQuant X, Media Cybernetics, Bethesda, MD) and rendered with ImageJ [11].

3. Results and discussion

After clarification, placenta tissue immunofluorescence was stable at room temperature for at least 3 weeks. As can be seen in Fig. 2, CD31 (red) staining of endothelial cells labels villous vessels and shows their complexity and diversity across early gestation (8, 12, 18 weeks) to term. Similarly, CK7 (green) labeling shows an intact trophoblast layer covering the villi. These images are suitable for extraction and analysis of villous structure and internal villous capillary structure. Examination of volumes revealed a complex arrangement of intermingled villous trees that resemble that seen in 2D histology slides and scanning electron microscopy [12–14]. Fig. 2 also shows a negative control image of unstained, cleared 22-week placental tissue. Some green autofluorescence is evident, but the near infrared autofluorescence is not detectable.

To date, the analysis of 3-D geometry of placental villous structure and capillary networks has been confined to all but the smallest and simplest terminal villi. We here present a novel method for clarification of larger volumes of placental tissue that can be then skeletonized, permitting analysis of complex villous structures and capillary networks at the order of 1 mm^3 of villous tissue in a matter of hours, compared to 2–3 weeks [15]. Tissue clearing generally involves one of three approaches: 1) uniform matching of refractive indices of tissue components by submersion in refractive index matching solutions, 2) removing lipid components by embedding in hydrogel and using electrophoresis/diffusion to remove lipids; 3) expansion/denaturation of protein structure to allow increased solvent penetration to encourage uniform refractive index. While these approaches are able to render tissues transparent and allow for 3D representations of structures, the clinical value of these 3D representations is problematic as it remains a challenge to determine if these images indicate tissue properties or artifacts of the clearing process. In addition, current techniques that are non-reversible preclude traditional histology.