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Redefining 3Dimensional placental membrane microarchitecture using multiphoton microscopy and optical clearing

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ABSTRACT

Introduction: Remodeling of human placental membranes (amniochorionic or fetalmembrane) throughout gestation, a necessity to accommodate increasing uterine volume, involves continuous alterations (replacement of cells and remodeling of extracellular matrix). Methodologic limitations have obscured microscopic determination of cellular and layer-level alterations. This study used a combination of advanced imaging by multiphoton autofluorescence microscopy (MPAM) and second harmonic generation (SHG) microscopy along with tissue optical clearing to characterize the 3Dimensional multilayer organization of placental membranes.

Methods: Placental membranes biopsies (6 mm) collected from term, not-in-labor cesarean deliveries (n = 7) were fixed in 10% formalin (native) or treated with 2,2′-thiodiethanol to render them transparent for deeper imaging. Native and cleared tissues were imaged using MPAM (cellular autofluorescence) and SHG (fibrillar collagen). Depth z-stacks captured the amnion epithelium, underlying matrix layers, and in the cleared biopsies, the decidua layer.

Results: MPAM and SHG revealed fetal membrane epithelial topography and collagen organization in multiple matrix layers. Term amnion layers showed epithelial shedding and gaps. Optical clearing provided full-depth imaging with improved visualization of collagen structure, mesenchymal cells in extracellular matrix layers, and decidua morphology. Layer thicknesses measured by imaging corroborated with histology. Mosaic tiling of MPAM/SHG image stacks allowed large area visualization of entire biopsies.

Conclusion: MPAM-SHG microscopy allowed for study of this multi-layered tissue and revealed shedding, gap formation, and other structural changes. This approach could be used to study structural changes associated with membranes as well as other uterine tissues to better understand events in normal and abnormal parturition.

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1. Introduction

Placental membranes (amniochorionic membranes or fetal membranes) are the innermost lining of the intrauterine cavity that protect the fetus during pregnancy. These avascular membranes are composed of simple cuboidal amnion epithelium, multilayered extracellular matrix (ECM), and the chorion, which connects to the maternal decidua [1,2]. Membranes are fully formed by week 15th; however, undergo constant remodeling throughout gestation involving collagen metabolism to maintain structural and functional homeostasis [3].

The amnion layer is the most elastic component of the placental membranes, and its remodeling is essential to maintain membrane integrity. During the remodeling process throughout gestation, epithelial cells are likely shed and replaced and the basement

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membrane is replaced with nascent type IV collagen [4]. A shedding cell in a tissue is defined by the loosening of its junctions with its neighboring cells and basement membrane, leading to its expulsion and the development of a gap [5]. These gaps appear as areas of smooth basement membrane void of cellular components. Shedding and gap formation are well reported in intestinal epithelial cells [5,6] as a part of remodeling of this tissue, and overwhelming shedding is linked to pathologic conditions, such as Crohn's disease [7]. We speculate that epithelial cell shedding, gap formation and resealing with new cells and nascent collagen are a part of tissue remodeling throughout pregnancy. Previous imaging studies using fetal membrane tissues after term deliveries have not reported well-defined shedding or gap formation, possibly because they are unlikely to be detected using thin 4–10 μm single-plane transverse views in histological or immunohistological methods. Limitations of advanced microscopic approaches (e.g. high-resolution imaging like confocal fluorescence microscopy or optical coherence tomography) also make it hard to study shedding and gap formation.

To overcome some of the limitations of current methodologies and better understand shedding, gap formation, and other structural changes to the membrane, we used the Nonlinear Optical Microscopy (NLOM) method of multiphoton autofluorescence microscopy (MPAM) and second harmonic generation (SHG) microscopy. This approach provides imaging of tissues to hundreds of micrometers in depth, with a subcellular resolution comparable to confocal microscopy [8]. A significant advantage of MPAM and SHG is that they can provide label-free images of cells/tissues through the collection of intrinsic signals (e.g., autofluorescence) [9], useful in cases where full depth labeling is a challenge. Optical clearing (OC) is a process that renders tissue optically transparent and can be used to extend imaging depth of microscopy. A variety of OC protocols have been reported [10] [11,12], though to our current knowledge, no studies of optical clearing in the placental membrane have been reported.

Primary objective of this study was to visualize and study membrane architecture at term fetal membranes using MPAM/SHG to better understand structural changes. Additionally, OC was used to render tissues optically transparent/clear to extend the depth of imaging by microscopy. MPAM/SHG with and without OC was used to reconstruct the multilayered 3-dimensional micro-organization of human placental membranes without the use of exogenous contrast agents.

2. Materials and methods

Placental samples were obtained for this study from John Sealy Hospital at The University of Texas Medical Branch (UTMB) at Galveston, TX, USA under a discarded tissue IRB (institutional review Board) protocol. The study protocol was submitted and approved by the IRB at UTMB (UTMB, Galveston, TX; protocol 11-251).

2.1. Fetal membrane collection for multiphoton microscopy analysis

Placental membranes (combined amniochorion and decidua) were collected from term not in labor (TNIL) cesarean deliveries ($n = 7$) with no documented pregnancy complications. Placental membranes were dissected from the placenta, washed 3 times in normal saline, and cleansed of blood clots using cotton gauze. Six-millimeter biopsies (explants) were then cut from the midzone portion of the membranes, avoiding the regions overlying the cervix or placenta. Explants were stored in 500 μL of 10% formalin in 1.5 mL Eppendorf tubes until imaging.

2.2. MPAM/SHG of membrane explants

In this study, the combined methods of MPAM and SHG were applied to investigate the multilayered 3-dimensional micro-organization of human placental membranes without the use of exogenous contrast agents. The primary fluorophores collected in MPAM are the reduced form of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotides (FAD) [9]. SHG arises from noncentrosymmetric molecules, including fibrillar collagen of the ECM, and has been applied for evaluating ECM organization in the placental membranes [13–17], but has not been combined with MPAM to our current knowledge. MPAM and SHG were conducted using a Prairie Ultima IV (Prairie Technologies/Bruker, Middleton, WI) upright microscope using excitation from a Ti:sapphire femtosecond laser (Mai Tai, Spectra-Physics, Santa Clara CA) (Fig. 1A). A 25× objective with 1.05 N A. (XLPlanN, Olympus, Tokyo, Japan) was used for image collection. Samples were illuminated at 840 nm for generation of both MPAM fluorescence and SHG, collected in a backscattering (epi-illumination) geometry. A 500-nm dichroic mirror in the detection path split autofluorescence emission (>500 nm) from SHG (centered at 420 nm), and MPAM/SHG were collected simultaneously in a 2-channel configuration. GaAsP PMT detectors were used (Hamamatsu, Japan). For mounting, the tissue was placed with the amnion side facing a #1.5 cover glass in a mounting chamber (30-mm cage plate—ThorLabs, Newton, NJ), with a second cover-glass placed on the chorion side; 300 μL of PBS was added to the tissue, and the cover slips were tightened to secure the PBS and tissue in the imaging chamber. The amnion layer was then oriented toward the objective on the upright microscope and centered with the help of an aiming beam from the excitation source. Regions of interest (ROI) were obtained (1s per frame; 512 × 512 pixels). Depth scans were obtained using a z-interval of 1 μm, with an imaging depth ranging from 110 to 400 μm (Fig. 1D). Images were obtained using a digital zoom factor of 1.19 resulting in a field-of-view of 408 × 408 μm. Image stacks were analyzed for epithelial characteristics, including morphology, shedding, gaps, thickness of layers, and collagen alterations using ImageJ/FIJI (NIH) software, while IMARIS (Bitplane, Concord MA) was used for 3D reconstructions of MPAM/SHG stacks (Fig. 1E). Imaging of the full explants in some samples was accomplished using image mosaic tiling (Fig. 1B). In these cases, z-stacks were obtained using a 10 × 0.3 N A objective (UPlanFI, Olympus) with a z-interval of 2 μm. An overlap region of 10% was used between acquired z-stacks (tiles) of the mosaic for optimal stitching between neighboring regions (Fig. 1C).

2.3. MPAM/SHG mosaic tiling of full intact explants

We characterized the amnion layer with MPAM and SHG and specifically examined cellular and matrix morphology characteristics (at the epithelial connective tissue interface, then beyond into the matrix layers). Besides imaging of innate placental membranes, we explored the possibility of extended depth of imaging after OC. Using intact placental membranes, we optically cleared the tissue with 2,2′-thiodiethanol (TDE; described below). We employed mosaic tiling as employed similar to the Tadokoro study, in which mosaic tiling was applied to the uterine horn [18]. For acquisition of images to encompass the full lateral dimension of intact explants, z-stacks were taken across a predetermined grid of the sample using the 10× objective, with neighboring regions sampled in a mosaic configuration (Fig. 1B). The mosaic was reconstructed using a custom algorithm. An overlap of 10% (controlled during acquisition) was used between neighboring fields for optimal stitching of adjacent regions. The grid was initiated at the upper left corner, and it progressed in the positive “x” direction. A grid size of 5 × 5

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