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A standardized method for collection of human placenta samples in the age of functional magnetic resonance imaging

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Abstract

Current methods for placental tissue collection assess a delivered organ without direct functional correlates; therefore, the four-quadrant biopsy protocol utilized by many researchers may provide reasonable representation of tissue across a large organ, and offer a snapshot for molecular analysis of the placenta. However, the recent impetus to understand the placenta in real time, and the use of functional imaging to comprehend placental biology, warrants a different sampling approach. Here we present a method to standardize placental tissue collection in a format designed to facilitate correlation of *in vivo* function with *ex vivo* assessments. Additionally, we draw comparisons to the quadrant biopsy regimen, and highlight a pathological case of placental infarction detected by *in utero* imaging.

METHOD SUMMARY

We describe a standardized method for the collection of placental tissue samples along the long axis of the placenta orientated around the umbilical cord insertion site. Use of the longest axis maximizes the number of sample sites and inclusion of the cord aids in anatomical orientation to correlate *in vivo* imaging data with *ex vivo* tissue-based function and histopathology.

Keywords

biopsy; placenta; standardized sampling

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AUTHOR CONTRIBUTIONS

VHJR conceived the study design, created the collection protocol, harvested samples, wrote and edited the manuscript. JEG assisted with sample collection, performed protein analysis and reviewed the manuscript. KSL assisted with protocol design and reviewed the manuscript. MCS performed the MRI scans and reviewed the manuscript. TKM assisted with study design, performed histology assessments and edited the manuscript. AEF conceived the study design and edited the manuscript.

The placenta is a heterogeneous and complex organ that performs a diverse range of functions as it develops dynamically across gestation. Much valuable knowledge has been gained from placenta studies performed post-delivery, as the tissue is readily available for *ex vivo* functional assessments [1]. However, the delivered placenta only provides a snapshot in time for tissue-based function and histopathology. What is needed is an approach to link *in vivo* functional data with *ex vivo* placental assessments. Uteroplacental blood flow is a primary factor determining maternal–fetal exchange, thus influencing fetal growth and development. We and others have demonstrated regional perfusion differences across the placenta that are likely to impact placental health and function [2-4]. However, the ability to meticulously examine uteroplacental perfusion *in vivo* was hindered by inadequate imaging modalities until recently. The NIH Human Placenta Project is the driving force behind recent significant advancements in placental imaging and real-time functional assessment capabilities [5]. Specifically, functional imaging of placental blood flow is generating unique data resources that provide comprehensive *in vivo* characterization of pregnancies, in addition to documented clinical outcomes [4,6,7]. In turn, the next step is to derive a representative and reproducible comprehensive placental sampling regime to maximize the potential for correlative *ex vivo* studies.

MATERIALS & METHODS

Imaging studies

This protocol was approved by the Institutional Review Board at Oregon Health and Science University (IRB#15196) with written consent obtained from all study participants. The study protocol in brief: women participated in three blood oxygenation level-dependent (BOLD)-MRI scans performed longitudinally across gestation on a Siemens Magnetom TIM Trio 3T whole-body system scanner, with placental tissue collection at the time of delivery (Figure 1).

Placental tissue collection protocol

Ideally, placental dissection should begin immediately following delivery. First, trim the fetal membranes, collect a 3 × 3-cm sample in foil and snap freeze. Using the schematic in Figure 1B, locate the longest axis of the placenta that includes the insertion of the umbilical cord, as these anatomical features will be used for orientation. If the umbilical cord is peripheral (rather than central insertion), orientate the placenta with the cord to the left-hand side of the longest axis. Mark the placenta by cutting ~1 inch into the tissue on the right-hand side (upper and lower cuts) and the lower left side. The upper left cut should be ~2 inches to distinguish it and aid re-orientation. Photograph both sides of the placenta with a ruler in the image. Cut off the umbilical cord, leaving approximately 0.3 cm attached. Collect 10 cm of umbilical cord closest to the insertion point and snap freeze in foil. Weigh the trimmed placenta, measure and record dimensions (X being the longest axis, Y the perpendicular measure and Z the tissue thickness; Figure 1B). Use the orientation incisions to cut a ~1.5-cm strip along the full length of the placenta cutting either side of the umbilical cord. Lay the strip on the second dissection scheme (Figure 1C or D, depending on cord insertion location), with the chorionic plate and the basal plate facing the top and bottom

edges respectively, photograph and determine the number of sampling sites. Starting with the central section, make full-thickness cuts to dissect the placenta per the schematic (Figure 1C & D). Systematically collect samples from the cord to the far-right peripheral site followed by cord to left for consistency in timing.

At each sample site, full-thickness tissue should be collected in cassettes for formalin fixation (Figure 2). Depending on placental thickness, tissues may need to be secondarily cut to ensure adequate tissue penetration of fixative (Figure 2B & C). Maintain correct orientation within each cassette and label as 'M' (maternal side) and 'F' (fetal side) as appropriate (Figure 2). For villous RNA and protein studies, dissect off the chorionic plate (placental part of the fetal membranes) and maternal decidua to leave isolated villous tissue for banking (Figure 2D). Further dissect the villous tissue into eight pieces and distribute using a checkerboard approach (Figure 2E) into two pre-labeled foils (for RNA and protein analysis, see Figure 2F), snap freeze all tissues in liquid N_2.

Quadrant biopsy sampling

As a proof-of-principle study, we also performed standard four-quadrant tissue sampling in three study participants (Figure 1B labels Q1–4) for comparative protein analysis. Villous tissue pieces (~2 g) were crudely dissected from the maternal-facing side of the placenta and snap frozen in liquid N_2. Full-thickness tissue sections were also placed in cassettes, fixed in 10% zinc formalin for 48 h and paraffin embedded for histological assessment. These quadrant biopsies were taken immediately prior to the dissection and processing of the individual sample sites collected from the orientated stip.

Protein analysis

To test for variance in placental protein from different sampling sites (by orientated strip vs quadrant methods), villous tissue was homogenized in lysis buffer containing protease inhibitors and centrifuged at 20,000×g for 5 min, as previously described [8]. Protein quantification was determined by BCA assay (Bio-Rad). Protein samples (20 μg) were heat denatured at 95°C for 5 min, separated on 10–20% Tris-glycine pre-cast gels (Invitrogen), transferred to nitrocellulose membranes using the iblot dry transfer system (Invitrogen), and probed by standard Western blotting for anti-GLUT4 (1:1000, Abcam) and anti-HSP70 (1:1000, Cell Signaling Technology) as markers of transport function and oxidative stress, respectively. Blots were reprobed with the housekeeping protein anti-Actin (1:1000, Abcam) to normalize for protein loading.

Placental histopathology

Placental tissue samples were evaluated for gross and/or microscopic infarctions using formalin-fixed, paraffin-embedded histological sections (5 μm) stained for hematoxylin and eosin by a placental pathologist (TKM).

RESULTS & DISCUSSION

Generation of biobanks and tissue repositories requires careful consideration with advanced planning to ensure optimum sample collection and appropriate preservation for subsequent

use [9,10]. It has been previously demonstrated that there are significant gene expression differences between the center and periphery of the placenta [11]. More recently, we and others have shown that regional uteroplacental perfusion differences occur in a non-uniform manner, independent of central versus peripheral location [2-4]. Understandably, differences in blood flow could lead to discrepant findings in histopathology and function across the placenta if sampling strategies are inadequately representative of *in vivo* targets. Thus, with the development of more sophisticated placental imaging modalities, it is necessary to devise more accurate *in vivo/ex vivo*-orientated placental sampling protocols, which can be implemented in studies that utilize advanced imaging to assess placental function.

We orient the *ex vivo* placenta by the long axis and umbilical cord insertion (strip method). We have standardized sampling and collection (i.e., the order in which samples are obtained is consistently done from the cord site through to the right and then from the cord to the left periphery), and here we compared the strip method with the standard four-quadrant placental sampling approach. An important potential consideration of our new method could be time delay required for orientation and additional sampling. For quality assurance, to assess the effect of timing, and to compare the strip versus quadrant biopsy sampling regimes, western blotting was performed for GLUT4 and HSP70 (Figure 3) to test for differences in signal related to sampling delay from right to left or quadrant 1–4.

GLUT4 expression demonstrated some regional variability in both the strip (Figure 3A) and quadrant (Figure 3B) sample sites, and they were comparable in the pooled tissue samples (Figure 3C). Importantly, HSP70 as a measure of tissue oxidative stress did not show increasing expression with additional time taken for sample preservation (left was not greater than right by strip method and quadrant 4 was not greater than 1 by quadrant method in Figure 3D & E). Data appear largely reproducible between the three individuals using pooled protein samples (Figure 3F). In agreement with these protein integrity findings, standard assessment of hematoxylin and eosin-stained slides revealed no histopathologic differences between sampling locations by the strip or quadrant methods (data not shown).

In one additional study case, we detected an abnormality on imaging that appeared as a ‘hole’ in the placenta (Figure 4A & B). This region was carefully identified during the strip dissection post-delivery (Figure 4C) and, in addition to the standard biopsy collection, tissue samples were preserved for histopathological assessment (Figure 4D). A placental pathologist (TKM) identified this region as an acute lobular infarction.

In summary, the anatomically oriented strip method presented here accounts for tissue depth and proximity to maternal spiral artery inflow to the intervillous space maintaining histological architecture to examine terminal villi versus stem or anchoring villi that are exposed to a gradient in oxygenated blood. Collecting biopsies via the checkerboard scheme should account for functional differences that may be linked to variation in uteroplacental blood flow measured *in vivo*. Standardization of tissue collection per this cost-effective protocol should allow for sampling consistency and reproducibility between individuals responsible for tissue collection, as well as ensuring a manageable but sufficient number of biopsy sites collected from each placenta. Importantly, the use of the umbilical cord as an anatomical feature may allow for correlation between *in vivo* measurements with *in vitro*

tissue analyses, if the longest axis of the placenta can be identified during analysis of acquired imaging data (Figure 1A). In addition, consistent sampling of the umbilical cord, with free cord taken adjacent to the site of placenta insertion, and placental samples preserved for histological assessment at the central cord site in our strip method, allows for site-specific and directed analysis of fetal responses to the *in utero* environment. For example, expression of inflammatory markers of the fetal inflammatory response is known to vary along the cord, with the placenta insertion site being particularly susceptible to immune cell infiltration [12]. Accuracy in sampling sites, timing and tissue-preservation methods has allowed us to generate a reliable placenta biobank resource from our NIH Human Placenta Project cohort of longitudinally characterized normal and pathological pregnancies.

FUTURE PERSPECTIVE

With the current rate of advancement and application of technology to understanding placental development and function during pregnancy, post-delivery placental analysis must similarly advance in sophistication. The first and most crucial step towards the success of correlative *ex vivo* placental studies is appropriate sample collection, processing and preparation. The new method described here does not preclude site-directed biopsies for clinical assessment of histopathology, and may not be suitable for all placental studies, but it outlines an experimentally motivated tissue collection regimen for standardized sampling, with the potential for correlative functional analyses of real-time placenta imaging studies.

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FINANCIAL & COMPETING INTERESTS DISCLOSURE

NICHD Human Placenta Project U01 HD 087182 (AE Frias) and R01 HD 086331 (AE Frias). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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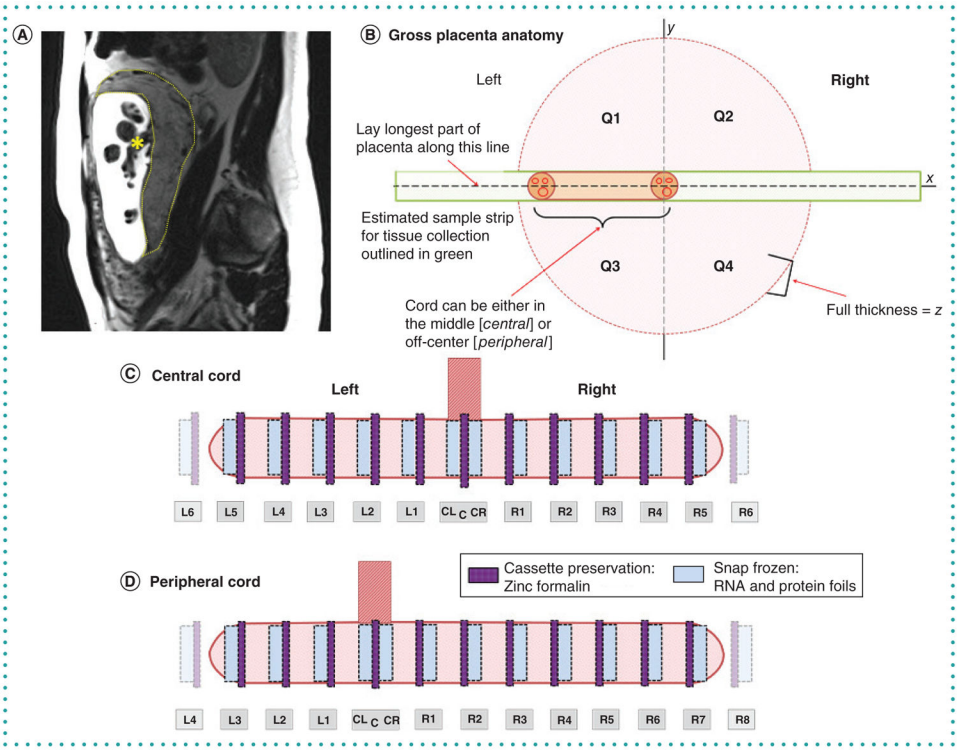

Figure 1.

Placental orientation and gross dissection schematic. (A) Sagittal view of a human MRI with the placenta outlined in yellow and the umbilical cord noted by the yellow asterisk. (B) Gross placenta schematic layout for orientation of the longest axis and dimensions for measurement. The four-quadrant biopsy sites are indicated by Q1–4. Dissection schematic for (C) central and (D) peripheral umbilical cord insertion locations. Full-thickness biopsy sites for cassette preservation in zinc formalin are indicated in purple, villous tissue biopsies are depicted in blue and red areas indicate excess tissue between sample sites. Note: the red portions are discarded once sample collection is complete.

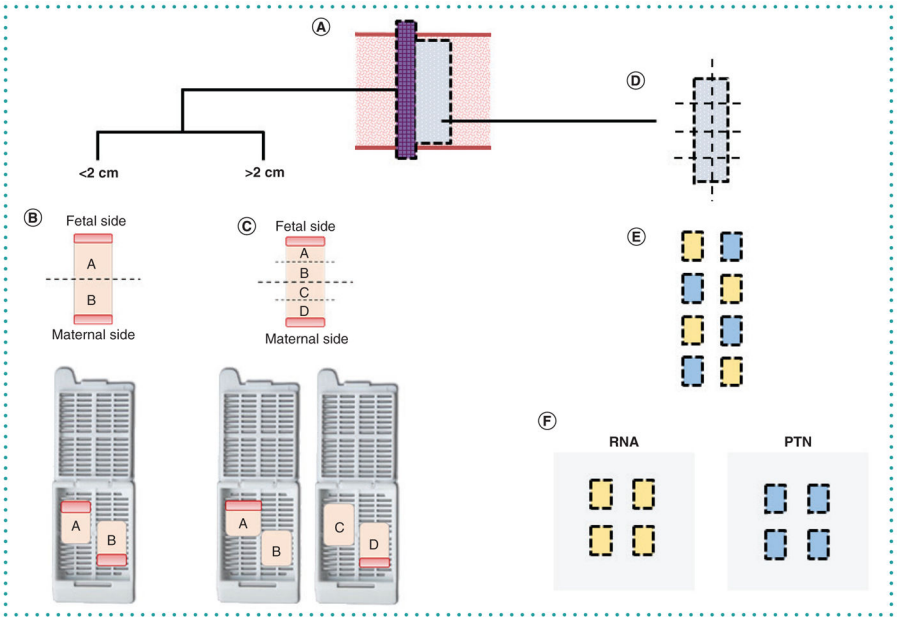

Figure 2.

Individual sample site biopsy schematic. (A) An individual biopsy site. Full-thickness tissue is dissected into (B) two pieces and collected in a single cassette when the tissue depth is less than 2 cm, or (C) four pieces and collected in two cassettes when the tissue exceeds 2 cm in thickness. Be careful to keep the tissue in the correct orientation. (D) Villous tissue biopsies should be further dissected into eight small pieces (E) and distributed by a checkboard pattern between (F) two foils for RNA and PTN analysis.

PTN: Protein.

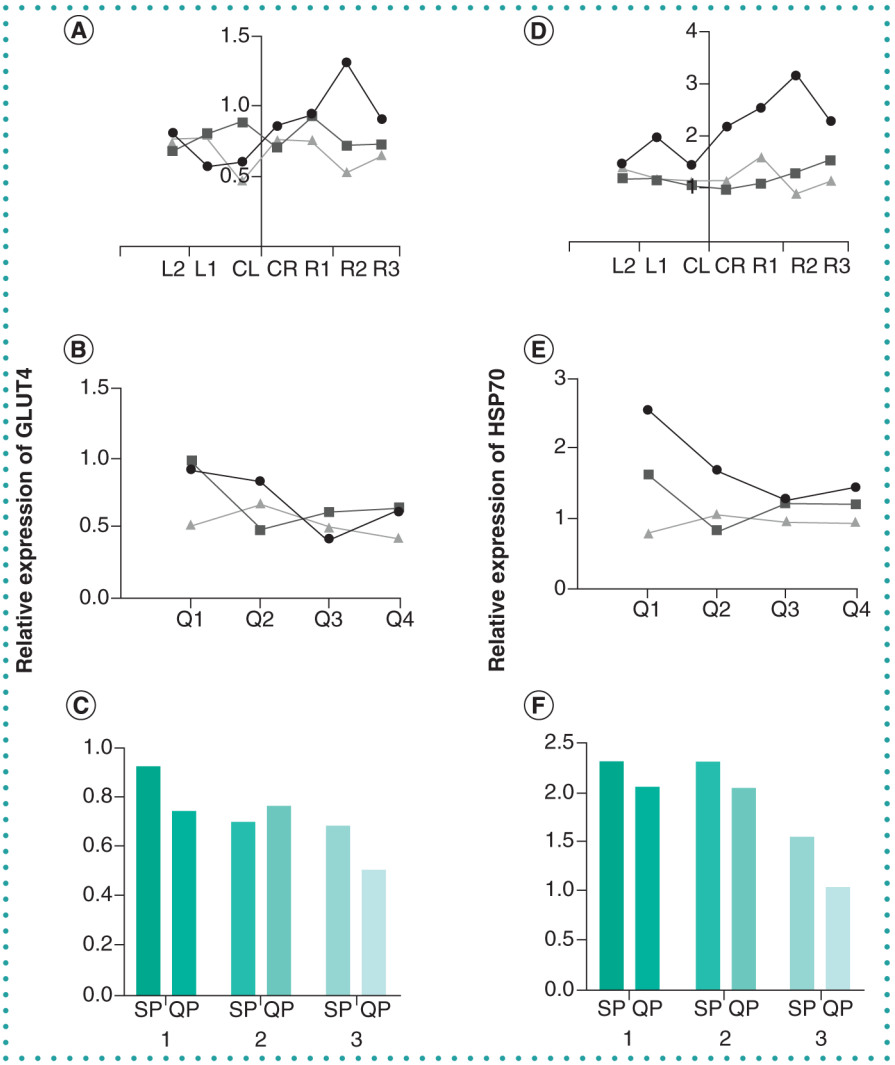

Figure 3.

Protein expression by collection method and location of sample. GLUT4 relative protein expression data in (A) strip versus (B) quadrant biopsy sampling and in (C) pooled samples from all sites in three individual placental collections. HSP70 relative protein expression data in (D) strip versus (E) quadrant biopsy sampling and in (F) pooled samples from all sites in three individual placental collections. CL: Cord left; CR: Cord right; L1–2: Samples dissected to the left of the cord; R1–3: Samples dissected to the right of the umbilical cord with R1 in closest proximity to the cord and R3 towards the periphery of the placenta; Q: Quadrant method pooled from 4 sample sites; Q1–4: individual quadrant biopsy sites; SP: Strip method pooled protein from all sample sites.

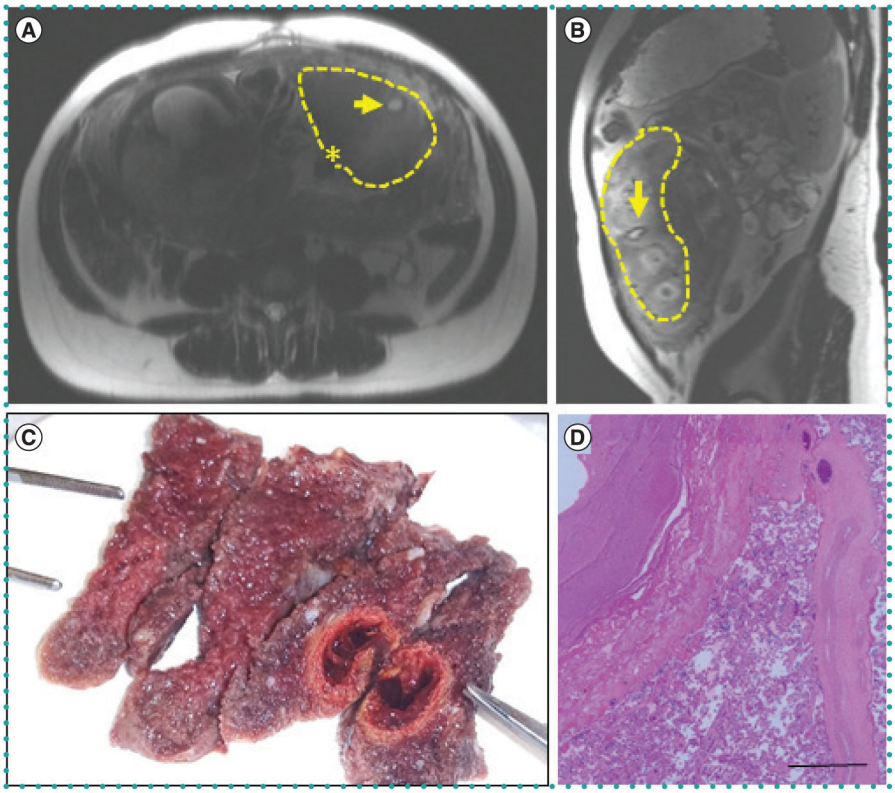

Figure 4.

Placental infarction identified by *in vivo* imaging and anatomically oriented *ex vivo* using the placental sampling strip method. (A) Axial and (B) sagittal T2-weighted MRIs where the abnormal area was visualized (yellow arrow; placenta is outlined in yellow and the yellow asterisk denotes the umbilical cord insertion). (C) Gross image of abnormal area, and (D) hematoxylin and eosin-stained image of the infarction. (D) Scale bar: 2 mm.