

Reviewer 2

General comment: In the manuscript "Three-Dimensional Histological Characterization of the Placental Vasculature Using Light Sheet Microscopy" Freise et al. set out to employ Light Sheet Microscopy to examine aspects of placental development and compare their observations to those derived using standard techniques. This exciting and pioneering study applies a new technique to examine critical aspects of placentation that may help researchers better understand fundamental biological processes involved in placental development and function. However, this paper needs to be better organized, tell a focused story, include critical methodological details expected of a technical paper, and present convincing evidence that this technique works. In its current form, this study is unsuitable for publication.

Response: We thank the reviewer 2 for taking the time to review our manuscript and appreciate your criticism and valuable feedback. We edited the manuscript upon your valuable suggestions and hope that it is now suitable for publication. Here, you can find our responses to your specific remarks:

Major comments

Comment 1: First and foremost, this is a technical paper. Therefore, the introduction needs to discuss confocal microscopy, highlight the relevance to studies of placental biology, emphasize the technical limitations of this technique, then introduce light sheet microscopy, explain this new technique, and contrast this form of microscopy with confocal so that readers can understand why the authors are conducting this work. The authors need to significantly abbreviate their discussions of placental biology and disease, then tell readers the story of light sheet vs. confocal microscopy.

Response: Thank you for this valuable comment. Indeed, an introduction of confocal microscopy, highlighting the advantages of lightsheet microscopy will straighten the storyline. Thus, we have added a respective paragraph in the introduction (L119-132). We understand the reviewer's view regarding the discussion. However, we have established our protocol specifically with the aim to apply light sheet microscopy to study lymphatic mimicry during spiral artery remodeling, i.e. in light of a specific biological question. Therefore, discussing the application of our results regarding the lymphatic mimicry is one of our main aims for this study. Consequently, a significant reduction of this part of the discussion would change the focus of our study. However, we hope that the addition to the introduction and further changes we have made following the reviewer's comments below help to clarify the story and aim of this study, which is to establish a light-sheet protocol to specifically study lymphatic mimicry in blood vessels of the mouse placenta.

Comment 2: This manuscript describes a novel approach to analyzing mouse placental development and function. Therefore, the authors need to measure established, well-characterized features of the mouse placenta and compare their resolution, reproducibility, and quantitative measures between confocal and light sheet techniques. Why did the authors choose this study to chase questions concerning the presence of the lymph system when the presence or function of this system in the placenta is debated? When validating a new protocol, readers want to compare established measures of histological organization, antibody staining, and architecture between the current (confocal) and new (light sheet) techniques. The authors must employ established antibodies and staining techniques and validate their staining using established 2D IHC. Otherwise, readers lack convincing proof that the author's report is accurate. This reviewer has enthusiasm for this work - but when developing a new

technique, go slow and use established measures to prove the validity and reproducibility of your observations.

Response: Thank you for pointing this out. The reviewer summarized correctly, that we aim to establish a new protocol to image placental tissue by using light sheet microscopy. However, while we understand the reviewers concerns, we established this method to target our specific biological question of the vascular remodeling and lymphatic mimicry. As pointed out by the reviewer, the presence of a lymphatic system in the placenta is still under debate [1–3]. However, Pawlak et al. using the traditional method of 2D immunohistochemistry have already shown, that the spiral artery endothelial cells start expressing lymphatic markers during the remodeling process [4], which was also confirmed by others [5]. Therefore, we consider this change in marker expression an established feature of the mouse placenta. To address the question of lymphatic system and lymphatic mimicry in more detail and on a spatial scale, new methods need to be applied. Here, we aimed to establish light sheet microscopy for this specific application. Importantly, lymphatic mimicry is potentially involved in the pathology of preeclampsia and we aim to use relevant rodent models to investigate this in the near future [5]. Therefore, using C57BL/6J mice here as a suitable healthy control group for establishing the protocol lays the foundation for future studies with preeclampsia mouse models.

Comment 3: Why did the authors cut the placentas into 1.5mm slices? The stated benefit of light sheet microscopy and the core premise of the paper is the need for detailed, non-destructive visualization of the placenta. There are several published examples of entire adult mouse brain and body clearing; why not here?

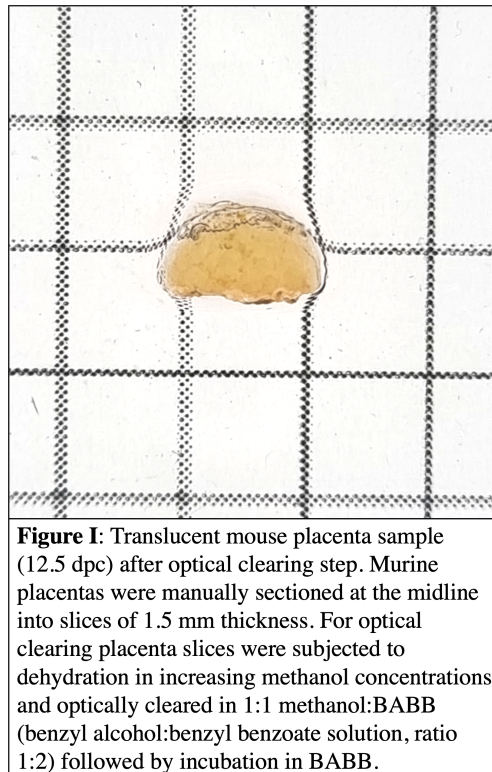
Response: Thank you for your valuable comment. The reviewer raised an important question. Indeed, there are examples of whole organ and even whole body clearing. Additionally, a whole organ staining, e.g. of an adult murine brain, has already been performed [6]. However, the tissue morphology of the placenta (dense tissue with only little fat) aggravates the staining and clearing process. As mentioned in the discussion our ex vivo staining approach is based on passive diffusion with IgG antibodies, thus the density of the sample limits the volume of the tissue. Furthermore, the placenta is well-supplied with blood. Erythrocytes present in a whole placenta tissue sample diffract the laser light, finally leading to “shadows” in the microscopic images. In previous experiments (not shown) we worked with different antibody-concentrations, sample sizes and incubation times. Based on this the conditions shown in the manuscript were chosen to be most suitable and the sample size used in our study represents the best compromise between the limitations mentioned and relatively large sample size. However, as mentioned in the limitations paragraph, we are currently working on imaging whole organs by the usage of nanobodies and the introduction of a bleaching step, thus facing all the minor limitations of the current approach (L492-496).

Comment 4: Why did the authors not use DAPI staining to confirm antibody penetrance?

Response: Thank you for this question. In our case DAPI is unsuitable to compare the antibody penetrance for two reasons: First, the size of DAPI as a chemical fluorescent probe is about 500 times smaller than for the used IgG (0.277 kDa DAPI, ~150 kDa IgG) [7,8]. Hence, the penetration of DAPI will significantly outrun the penetration of the antibodies. Second, DAPI gets excited at 358 nm, in contrast, the shortest excitation wavelength of the used Zeiss LightSheet7 is 445 nm. Thus, DAPI is unsuitable for our experimental set up and was replaced by acridine orange featuring the same nuclear staining properties like DAPI. As shown in the new Figure 1, acridine orange confirms penetration of the entire sample.

Comment 5: The authors must include representative images to prove they achieved complete tissue clearing.

Response: Thank you for this comment. We kindly refer to a representative image of an optically cleared placenta sample shown in figure I.



Comment 6: The authors present multiple subjective observations as proof of antibody success and tissue penetration. However, readers need to see analysis using quantitative methods.

Response: Thank you for this comment. For this manuscript, we are staining for lymphatic markers, which are not ubiquitously expressed in the placenta. Therefore, a visual measurement of antibody penetration is not consistent and thus unsuitable for quantification. Additionally, we have chosen the size of the tissue samples to obtain full antibody penetration, which can be ensured by measuring the fluorescence-signal at the center of the sample and by the use of acridine orange as described above. This is also shown in the representative images (Fig. 1) in the manuscript. Furthermore, this study aims to assess the expression of the chosen markers qualitatively and does not aim to provide a quantification of expression levels.

Comment 7: Overall, the protocol could have been more specific and detailed. For example, what was the duration of immunostaining? How many placentas did the authors use? In addition, there needed to be more explanation for why the authors chose specific methods or solutions over other alternatives.

Response: Thank you for these important additions. We have amended the paragraph accordingly (L156-164).

Minor Points:

Comment 1: Figure 1. A1-C1 and A2-C2 should be switched in figure legend or Figure 1: C1 and C2 swapped.

Response: Thank you, the figure legend has been corrected.

References:

1. Becker J, Tchagou Tchagou GE, Schmidt S, Zelent C, Kahl F, Wilting J. Absence of lymphatic vessels in term placenta. *BMC Pregnancy Childbirth*. 2020;20(1):380. doi:10.1186/s12884-020-03073-w
2. Red-Horse K. Lymphatic vessel dynamics in the uterine wall. *Placenta*. 2008;29 Suppl A(Suppl A):S55-9. doi:10.1016/j.placenta.2007.11.011
3. Red-Horse K. Cytotrophoblast induction of arterial apoptosis and lymphangiogenesis in an in vivo model of human placentation. *J Clin Invest*. 2006;116(10):2643-2652. doi:10.1172/JCI27306
4. Pawlak JB, Bálint L, Lim L, et al. Lymphatic mimicry in maternal endothelial cells promotes placental spiral artery remodeling. *J Clin Invest*. 2019;129(11):4912-4921. doi:10.1172/JCI120446
5. Vogtmann R, Heupel J, Herse F, et al. Circulating Maternal sFLT1 (Soluble fms-Like Tyrosine Kinase-1) Is Sufficient to Impair Spiral Arterial Remodeling in a Preeclampsia Mouse Model. *Hypertension*. 2021;1(October):1067-1079. doi:10.1161/HYPERTENSIONAHA.121.17567
6. Stefaniuk M, Gualda EJ, Pawlowska M, et al. Light-sheet microscopy imaging of a whole cleared rat brain with Thy1-GFP transgene. *Sci Rep*. 2016;6(1):28209. doi:10.1038/srep28209
7. Janeway CA Jr, Travers P, Walport M et al. Immunobiology: The Immune System in Health and Disease. 5th edition. In: *Immunobiology: The Immune System in Health and Disease. 5th Edition*. Garland Science. <https://www.ncbi.nlm.nih.gov/books/NBK27144/>
8. PubChem Compound Summary for CID 2954, 4',6-Diamidino-2-phenylindole. https://pubchem.ncbi.nlm.nih.gov/compound/4_6-Diamidino-2-phenylindole