**Editorial comments:**  
Changes to be made by the Author(s) regarding the written manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

*Author’s response: As recommended we proofread the manuscript.*

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

*Author’s response: The link to the editorial policy of Frontiers Immunology that allows re-prints was uploaded via a doc file to the Editorial Manager account of JOVE. A specific citation appears in the figure legend*.

3. Please revise the title to be more concise.

*Author’s response: We have changed the title into: High frequency ultrasound for the analysis of fetal and placental development in vivo“*

4. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

5. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

*Author’s response: The revised version of the manuscript includes SI abbreviations for all units as well as a space between numbers and units.*

6. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Vevo 2100, FujiFilm VisualSonics Inc., MS550D-0421, etc.

*Author’s response: We thank the editor for this remark and removed all commercial language from the manuscript and used generic terms instead.*

7. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "Vevo 2100 Imaging System" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

*Author’s response: See comment number 6.*

8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

*Author’s response: The protocol text was revised and all personal pronouns were removed.*

9. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

*Author’s response: We thank the editor for pointing out this issue that was revised as recommended. Now every step of the protocol is written in imperative tense and additional information can be found in the “Note”-parts.*

10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.  
For example:  
1.5: Please describe how this step is actually done.  
3.1.2: Please specify how proper anesthetization is confirmed.  
*Author’s response: We added more details to our protocol steps to be sure that viewers can replicate the protocol easily.*

**Reviewers' comments:**  
Reviewer #1:  
Manuscript Summary:  
The protocol submitted by Meyer et al presents a detailed description of high frequency ultrasound in mouse fetuses. The protocol is well written and easy to follow, with adequate details for a reader to be able to perform a similar analysis. The images presented with the manuscript greatly aid in understanding the experimental setup, as well as the data acquisition and analysis. Overall this is an excellent protocol. However, there are a few minor concerns that could be addressed to further strengthen the manuscript.  
  
Minor Concerns:  
The authors mention in the discussion that a major strength of the protocol is that it may be applied serially throughout a pregnancy in order to follow the same fetuses through in utero development. Although the protocol is relatively non invasive for the animals, they still need to be anaesthetised and have their fur fully removed. This causes a stress on the animal, particularly with temperature regulation. Could the authors comment on any post-op support that is given to the animal to minimise this stress? Also, how does repeated anaesthesia and hair removal affect the physiology of the pregnancy in mice?  
*Author’s response: We thank the reviewer for this helpful comment. Indeed, the anesthesia causes stress to the animal. To reduce the stress level of the mice to a minimum we let the animal alone in a cage for a minimum of 5 min after the measurement. The animal has time to wake up and to orientate before we place it back in the original cage where it is housed together with other mice. Mice wake up very quickly after the isoflurane was turned off. It takes just around 20 s. Another point to reduce the stress is to do the ultrasound measurements not every day but not more than every second day (here gd5, 8, 10, 12, 14). We have now mentioned these important points in the protocol.*

*We do not think that the hair removal affects the physiology of pregnancy in mice. For the used depilatory cream there is no hint that hair removal should not be performed during human pregnancy. We cannot be sure that the repeated anesthesia does not affect pregnancy parameters in mice. But if so, it should have the same effect in the control group.*

It may be useful for readers who may be unfamiliar with the Vevo 2100 system to add some details about the sensitivity and reliability of the instrument. For example, approximately what magnitude of uterine artery velocities can be reliably resolved between groups? Also, approximately how many animals would need to be studied in order to find a statistically significant difference in various measurements?  
*Author’s response:*

*The UA velocity depends on different conditions, for example if the animal is pregnant or not pregnant, on the specific gestation day as well as the mouse model. In the literature you find several publications analyzing UA velocities at different stages or under different conditions (examples: PMID:16603699, 26811058, 25968580, 23986360, 29319186). To proof the reliability of the instrument it is conceivable to sacrifice a few animals at gestation days of interest and analyze specific parameters e.g. placental weight to compare these results with the data obtained by using the Vevo 2100 System. How many animals are needed for a study depends on the individual experimental design. For analyzing fetuses or placentas possibly fewer animals are needed because mice have high litter sizes.*

The authors also mention in the discussion that rather a high amount of ultrasound gel is needed to acquire high quality images. Please provide an approximate volume of gel needed per mouse.  
*Author’s response: We used an amount of around 10 ml gel per mouse and included this information in the discussion part.*

Reviewer #2:  
Manuscript Summary: This manuscript aims to describe the technique and utility of ultrasound during murine fetal gestational to obtain longitudinal fetal growth patterns and placental development. This will provide less invasive options for fetal monitoring and research.  
  
Major Concerns: No major concerns; well composed and easy to interpret  
  
Minor Concerns:  
- Needs minor language editing for better flow

*Author’s response: We thank the reviewer for this comment and proofread the manuscript as recommended.*

- Introduction should be more concise

*Author’s response: Thank you! We have now changed the introduction for more conciseness.*

- Ultrasound for mice is not "harmless" as anesthesia is required (carries small risks, pain of injection, etc)

*Author’s response: We agree with the reviewer and mentioned that there is a risk for the animal due to the isoflurane narcotization. To reduce the stress for the mouse because of the narcotization ultrasound measurements should be done not more often than every second day.*

- "below the bladder" is confusing - use anatomical direction (caudal/cephalad)

*Author’s response: We thank the reviewer for this valuable suggestion and changed to the correct anatomical directions.*

- How do you ensure the correct "transplacental" cross-section - if it is not midline, the placenta will be measured too small (or if the plane is not perpendicular, it will be measured too large)

*Author’s response:* Because of the high amount of fetuses within the belly and the limited space it is not easy to present the correct transplantal cross-section of all placentas and it takes a certain time. Nevertheless, f*or precise placenta measurements all implantations were positioned in the same way. Frames or cineloops were stored when the UmA blood flow could be seen. This position could not always be reached for all placentas, so that not every placenta from each animal may be measured but as many as technically possible.*

- Authors should note that MRI and CT have been used on pregnant mice, so ultrasound is not the only method  
*Author’s response: We thank the reviewer for this comment, agree and added the information to the manuscript.*

Reviewer #3:  
Manuscript Summary:  
This manuscript describes how the Vevo 2100 high resolution ultrasound imaging device works and can be used to study intrauterine growth restriction (IUGR), now called fetal growth restriction (FGR). The authors provide step by step procedural details to fire up the equipment and to prepare mice for this protocol. There is one major issue here. The manufacture of the device and other investigators have already described exactly the same details for assessing fetal blood flow, placental and fetal development, organogenesis, and tumors. The use of mice deficient in NK cells and mast cells may be an additional focus, although the rationale for NK and mast cell deficiency is not provided.  
*Author’s response: We thank you for this remark. Based on our previous publications (doi: 10.1038/srep45106, doi: 10.3389/fimmu.2017.01913. eCollection 2017.) we were invited from JOVE to write this manuscript about the ultrasound technique. As the journal focus on methods rather than on the background of the study itself, we didn’t provide the rationale for NK and MC-deficiency. By citation of our previous studies we give the reader the possibility to understand the rationale behind the use of this mouse model. The only important point to know for this paper is that NK/MC-deficient mice give birth to growth restricted pups. This is written in the text.*

*We agree with the reviewer in the point that protocols from the manufacture exist. The text presented here has been proofed and accepted by the company before submission.*

Major Concerns:  
1. The authors write in introduction that the method is cost-effective. Although the device is extremely useful, this comment may not be a correct one. The equipment costs close to $300,000 with minimum accessories. Adding specialized probes for different applications will balloon up the price to $400,000 or more. Finding an easily accessible space in the animal care facility is another issue. Since the device is likely to be used by multiple investigators, is there one technical operator for the groups to guarantee reproducibility?

*Author’s response: We thank the reviewer for this comment and agree. Although consumable materials that are needed for the technique are cheap, the machine and accessories are very expensive. We are sorry for the incorrect statement and changed it within the introduction part.*

*Concerning your comment regarding finding space in the animal facility or multiple investigator usage: Of course the acquisition of an ultrasound device it not possible or useful for every laboratory. Every group should decide if there is the space, if they have the money and if it fits with the experimental designs. The protocol written in the present publication should help investigators that already have such a machine to improve its use or it may help other researchers to evaluate whether the described method may be the optimal one for answering their scientific question. The sentence that says the measurements should be done by the same operator is just a hint not a requirement in order to minimize mistakes.*

2. It would have helped a reader if the authors distinguished the use of the device for FGR and FGR/preeclampsia. How can the FGR be uniquely studied under two scenarios?

*Author’s response: We thank the reviewer for this question. We didn’t try to distinguish for FGR and FGR/preeclampsia with the use of the device. This was done with other methods in our previous study. We used high frequency ultrasound imaging to ascertain the in utero growth of fetuses and placentas from NK/MC -deficient mothers that give birth to growth-restricted pups and to determine the time point at which IUGR starts. We also employed Doppler measurements to document blood supply to the fetus in females that were deficient for NK cells and MCs.*

3. Will anti-CD122 antibody treatment of wild-type mice show any effects on fetal growth as observed by The Vevo 2100 device (Figure 2)?

*Author’s response: The anti-CD122 antibody treatment of WT mice has no effect on the weight of fetuses at birth (doi: 10.1038/srep45106). The anti-CD122 antibody treatment of MC-deficient mice, as shown here, affects fetal growth from gd10 onwards (Fig. 2A) and leads to a significant lower fetal weight at gd14 (Fig. 4A) gd18, and also at birth (doi: 10.1038/srep45106).*

4. In Figure 3, how come the placental area in anti-CD122 mice is normalized or higher on gd 14? Are these fetuses of the same size at birth? Such concerns also apply to other figures.

*Author’s response: We thank the reviewer for the interest in our experiments. As the placenta reach the full development around gd16 in mice, it is possible that they catch up their growth. At gd14 fetal weight is lower in MC/NK-deficient mice in contrast to controls (Fig. 4A) whereas placental weight is comparable between the groups (Fig. 4B). At birth fetuses of anti-CD122-treated mothers have a comparable weight to fetuses of PBS-treated WT mothers. Fetuses of anti-CD122-treated MC-deficient mothers show an significant lower birth weight compared to fetuses of PBS-treated WT as well as anti-CD122-treated WT mothers (doi: 10.1038/srep45106).*

5. Several references from Drs. Adamson's and Karumanchi's labs are not included.

*Author’s response: We are aware that Adamsons and Karumanchis labs have many relevant and very interesting publications about preeclampsia, hypertensive disorders, and fetal weigh. Nevertheless, for this manuscript, none of them was relevant.*

Minor Concerns:  
1. Please provide some conceptual explanations of their finding, such as the exact pathways that are dysregulated during pregnancy by NK and Mast cell deficiency.

*Author’s response: We understand that the reviewer is interested in specific pathways and the background of our study. Nevertheless, JoVE is a methods-based journal. To the best of our knowledge, an exact explanation of the background would not be suitable for the journal. In the “Transcript template” it is written for specific parts:*

*- “Abstract: The abstract should focus on the method being presented rather than the results of a specific experiment.”*

*- “Discussion: The Discussion section of the article should be focused on the protocol and not the representative results. “*

*Please see our previous publication (doi: 10.1038/srep45106) for the exact pathways that are dysregulated during pregnancy by NK/MC-deficiency if you are interested in.*

*Nevertheless, if the Reviewer may be interested to read our original paper published in Scientific Report where these issues are addressed.*