To Editors and Reviewers:

Thank you so much for all the efforts. It has been a hard time during pandemic, and I sincerely apologize that we have struggled with lots of difficulties in this special time. We have carefully proofread and revised the manuscript according to comments, and hope that the current version meets the publishing criteria. Please feel free to let us know if you have any further comments, and we are happy to take any suggestions to make this study better.

Thank you so much again.

Sincerely Yours,

Mingxiao Feng

Reply to Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   Authors' reply: Thank you for the comment. We have carefully proofread thoroughly the manuscript and corrected spelling/grammar issues.

2. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

Authors' reply: Thank you for the comment. A sentence for overall goal statement has been added into Introduction: "The overall goal of this protocol is to provide a fast and labor-efficient method to isolate primary hepatocytes from mouse, without jeopardizing the isolated primary hepatocyte purity and viability."

b) The rationale behind the development and/or use of this technique

Authors' reply: Thank you for the comment. We added the stressing of the rationale behind the development and use of this technique in second paragraph of the Introduction.

c) The advantages over alternative techniques with applicable references to previous studies

Authors' reply: Thank you for your comment. We have included this information in the second paragraph of the introduction as well as the discussion.

d) A description of the context of the technique in the wider body of literature

Authors' reply: Thank you for your comment. We have included a description of wider body of literature: "Liver serves as one of the most important organs in vertebrate body, due to a vital role it plays in numerous life-supporting functions like food digestion, blood circulation, and detoxification. Usage of mouse primary hepatocyte *in vitro* culture is increasingly popular in studies of carbohydrate metabolism and hepatic carcinoma. It is therefore important to develop a convenient method for mouse primary hepatocyte isolation while maintaining its innate physiological function.", and have more deeply discussed the developed technique in context of the wider body of literature in the discussion.

e) Information to help readers to determine whether the method is appropriate for their application

Authors' reply: Thank you for the comment. We have added information about the characters of this protocol, and the differences with previous protocols, in order to help reader to determine whether this protocol is useful for various application in the Introduction: "In the development of present protocol, efficiency was set as priority, with all reagents ready-to-use and available from market, and as few steps as possible. The overall goal of this protocol is to provide a fast and labor-efficient method to isolate primary hepatocytes from mouse, without jeopardizing the isolated primary hepatocyte purity and viability.", and in the Discussion.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
   Author's reply: Thank you for the comments. We have revised the text to avoid the use of such pronouns.

4. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
   Authors' reply: Thank you for the comments. We have added an ethics statement before numbered protocol steps: "All procedures were approved by the Johns Hopkins Animal Care and Use Committee."

5. Please mention how animals are anesthetized and how proper anesthetization is confirmed.
   Authors' reply: Thank you for the comments. We have added the method to anesthetizing and confirming: "7. Prepare 2ml 1X Anesthetics by mixing 225µl Ketamine HCL, 93.75µl Xylazine, and 1681µl 1x PBS.
   8. Anesthetize one mouse with proper method. Here the mouse was intraperitoneally injected with 150µl 1x Anesthetics. Tests for loss of reflexes such as reaction to toe pinching need to be done to endure full anesthesia."

6. Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

Authors' reply: Thank you so much for your comment. Because this isolation protocol is fatal to mice, as it removes the entire liver, the application of vet ointment on eyes is deemed not necessary.

7. What happens to the mouse after?
   Authors' reply: Thank you for your comment. This primary hepatocyte isolation protocol is fatal to the mouse because of removed blood volume and the essential organ of the liver removed.

8. For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.
   Authors' reply: Thank you for your comment. This primary hepatocyte isolation protocol is fatal to the mouse because of removed blood volume and the essential organ of the liver removed.

9. Discuss maintenance of sterile conditions during survival surgery.
   Authors' reply: Thank you for your comment. We have added the description of maintaining sterile conditions during primary hepatocyte isolation: "The maintenance of sterile conditions also plays an important role in this step. If the condition permits, it is better to conduct perfusion in a clean hood. All the tools with direct touch to tissues should be sterile, which can be achieved by autoclave. In order to prevent any contamination from bacteria and fungi, Antibiotic Antimycotic Solution (Sigma-Aldrich, Cat. A5955) was added into culture medium" in Discussion.

10. Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.
    Authors' reply: Thank you for your comment. This primary hepatocyte isolation protocol is fatal to the mouse because of removed blood volume and the essential organ of the liver removed.

11. Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.
    Authors' reply: Thank you for your comment. This primary hepatocyte isolation protocol is fatal to the mouse because of removed blood volume and the essential organ of the liver removed.

12. If the mouse is euthanized, please specify the method.
    Authors' reply: Thank you for your comment. This primary hepatocyte isolation protocol is fatal to the mouse because of removed blood volume and the essential organ of the liver removed.

13. Please upload Table 1 as an xls/xlsx file.
    Authors' reply: Thank you so much for your comment. The Table 1 was uploaded as .xlsx file.

Reply to Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The method presented by Feng et al., is a detailed easy-to-do method for mouse hepatocytes isolation that attempts to reduce time and effort during the process of isolation. The labor is intended to be reduced mainly by purchasing commercial media for perfusion and digestion processes whereas time was shortened by reducing 2 centrifugation steps based in previous reports. The protocol is clear and well described; all material are justified and indicated. The authors compared their presented method with methods reported previously throughout the manuscript and described clearly the characterisation of primary hepatocytes at different times of culture. Characterisation encompassed from specific markers to functional assays and results are overwhelming. Finally, the authors discussed the method's efficiency by approaching perfusion and purification steps.

Major Concerns:

1. The authors mentioned that primary hepatocytes are binuclear (diploid), however, they justified binuclear hepatocytes as a signature of hepatocytes differentiation. Morales-Navarrete et al. (A versatile pipeline for the multi-scale digital reconstruction and quantitative analysis of 3D tissue architecture) in 2015, demonstrated that diploid (or polyploid) mouse hepatocytes are enriched at the middle of liver sinusoid; mononuclear hepatocytes are present at the borders where hepatocytes can proliferate and it is dependent on the stimulus. Binuclear hepatocytes can not further replicate. I suggest the authors to include discussion about this because this new method could enrich this type of hepatocytes but no quantification is shown.

   Author's reply: Thank you for your comment. We have updated this part to include this information: "Based on the nucleus morphology of mouse hepatocytes, within liver, mononuclear hepatocytes were enriched at borders, while binuclear/polynuclear hepatocytes, signature of terminal differentiation, were in the middle. A significant amount of cells imaged displayed typical dual-nucleus (diploid) morphology, indicating the success of isolating and purifying of live primary hepatocytes.."

2. At the same paragraph the authors mentioned that there are not evident differences at the attachment of isolated hepatocytes between collagen-coated and collagen-free plates. I consider if the authors mentioned this data they should show a representative comparison. At the end the authors discussed properly that the plates they used could promote adhesion due to their fabrication features regardless of a deposition of a ECM protein.

   Authors' reply: Thank you for your comment. We have revised this part to conclude that "coating plates with collagen is not a requirement for primary hepatocyte attachment", instead

of directly comparing collagen-pre-coated plates with non-coated ones. Given the performance of our primary culture in our representative experiments, direct comparison is not necessary.

3. Characterisation of primary hepatocytes was based on RT-PCR method for positive and negative markers as for pharmaceutical biomarkers. Nevertheless, the authors did not discussed about the likelihood of that transcripts are reflected as translated proteins. Authors may discuss whether in other methods (ASGR1 protein levels are shown in reference 2) or reports, these proteins were detected or indicate that further protein characterisation is needed (immunoblotting).

   Authors' reply: Thank you for your comments. We have added discussion about protein level checking in the Results, section "Activity of pharmaceutical biomarkers was preserved": "It is worth noting that RNA and protein levels may be inconsistent, because of influences from post-transcriptional activities like signal peptide-induced RNA migration, posttranslational modification and/or protein degradation. Therefore, protein level and bioactivity verification of pharmaceutical biomarkers identified by mRNA may be necessary if required by the experiment paradigm."

4. At the section of glucose production, it is mentioned "gluconeogenesis/glycogenolysis studies" but only glucose production and phosphorylation of PEPCK (gluconeogenesis) are evaluated. It is likely that glycogenolysis is promoted but no evidence was presented so I suggest that authors should mention this as a high possibility. Gluconeogenesis, glycogenolysis and glycolysis are zonated in liver and it is dependent strongly on the activation of HNF4alpha and beta-catenin transcriptional factors among others (Colnot and Perret. Liver Zonation. Molecular Pathology of Liver Diseases 2011; Jungermann. Metabolic Zonation of Liver Parenchyma: Significance for the Regulation of Glycogen Metabolism, Gluconeogenesis, and Glycolysis 1987), I consider that it is important to discuss because the metabolic phenotype of primary hepatocytes is focused in glucose metabolism and important functional markers were not evaluated or discussed such as HNF4alpha.

   Authors' reply: Thank you for your pointing this out. We have added a disclaimer that we did not specifically investigate the cells' performance in experiments of glycogenolysis in the representative results, and have also added this as a limitation in the discussion this part in glucose production section.

Minor Concerns:

1. The authors should compare overall times between their method and the previous methods to highlight the reduction in time as it is mentioned in the title and highlighted along the manuscript.

Authors' reply: Thank you for your comment. We considered discussion comparing the times. However, because of different equipment logistics and experience of each user, the estimated times may vary. Instead, we emphasized that present protocol can be done within ~35min, and with fewest steps of published protocols, indicating it is of high possibility that present protocol takes the shortest time to complete.

2. The authors discussed bath temperature settings from 40 to 42ºC and decided to use an optimised temperature of 45ºC arguing the laboratory conditions and perfusion set-up, however, the authors should mention about the particular features of their set-up (room temperature, length of tubing, temperature in the outlet, etc.) that could help much for establishing conditions in new lab groups.

   Authors' reply: Thank you for your comments. We have added the room temperature information into the "Preparation" section of the manuscript: "Results are most reliable if the room temperature is at 25 °C.".

Reviewer #2:

Manuscript Summary:

Primary mouse hepatocyte isolation is a fundamental technique to investigate the hepatocyte physiology and function ex vivo. This study shows a relatively convenient way to isolate the primary hepatocytes and compare the method with already reported protocols. This proposed protocol is not so innovative, but there are some improvements compared to other protocols. Therefore, this study would provide informative information to the hepatocyte research community. However, the current manuscript needs some revisions.

Concerns:

1. Please include information about methods of anesthesia and euthanasia appropriately.

   Authors' reply: Thank you for your comment. We have added anesthesia information in the "Preparation" of "Protocol" section: "Anesthetize one mouse with proper method. Here the mouse was intraperitoneally injected with 150µl 1x Anesthetics. Tests for loss of reflexes such as reaction to toe pinching need to be done to ensure full anesthesia.

2. The type of collagenase was not clearly stated. Please add this information to the method, discussion, and/or Table 1. In addition, the pH of the perfusion buffer is also important. Please add the pH information.

   Authors' reply: Thank you for your comment. Both the collagenase and perfusion buffer were purchased. The Liver Digest Medium contains collagenase, and is available at Gibco with Cat.

No.: 17703-034. The Liver Perfusion Medium is available with “Gibco, Cat. No. 17701-038”, and there catalog numbers were provided in manuscript so that users can access the pH and type of collagenase provided by the manufacturer.

3. The authors use Liver Digest Medium, Gibco (500 mL). Does the medium have to be used freshly? (do the authors open a new bottle in every isolation experiment?). Or can the unused medium be used in the future? If so, how long of a period can the unused medium in the bottle be maintained in a refrigerator and be used for the hepatocyte isolation? This information would be helpful for the readers.

   Authors’ reply: Thank you for your comment. The Liver Digest Medium was aliquoted to 25mL each and keep in -20 degree freezer. It can be used until the expiring date marked by manufacturer. Each time 25ml Liver Digest Medium was shawled and used. We have included this information in Materials part : ”Aliquot within tissue culture hood to 25 mL each in 50 ml tube, and keep in -20 oC freezer”.

4. How many seconds and how many times did authors press portal vein during perfusion? Do the authors peel the liver membrane after the perfusion?

   Authors’ reply: Thank you for your comment. Every minute the portal vein was pressed throughout the perfusion and digestion process. The liver membrane was not peeled. However, in the step of filtering through 70 um filter, most of undigested liver membrane was filtered out.

5. The authors mix cell-containing media and percoll buffer, which may result in contamination of cells. In many protocols, cell-containing media is loaded on the percoll buffer, and then centrifuged, to avoid the mix. Do authors try the gradient method? Please include information (either in the method/discussion or in Table. 1).

   Authors’ reply: Thank you for your comment. To prevent this, we have included the method of preparation of 1 x Percoll-HBSS. The 1 x Percoll-HBSS used in present protocol was prepared in clean hood free of pathogens with purchased Percoll and 10 x HBSS. Percoll (Cat. 12-0891-01, GE Healthcare) is sterile (Ref.: http://www.ebiotrade.com/custom/GE/120220tw/images/D3.pdf, page 77), and so is 10x HBSS (Cat. 14065-056, Gibco) (Ref.: https://www.thermofisher.com/order/catalog/product/14065056#/14065056, on label). In this case, well-prepared 1 x Percoll-HBSS should not be the cause of contamination.

6. In Table 1, please include additional information about experiments done with the primary hepatocyte and/or applications used in each study. For instance, in this current protocol, glucose metabolism (pAKT, pFOXO1, glucose production) was investigated with the isolated

hepatocytes. Similarly, please add specific experiments/applications done in each study to the Table 1.

Authors' reply: Thank you for your comment. Since not all protocols compared in this study tested isolated primary hepatocytes at protein and metabolism level, and primary hepatocytes isolated in each protocol were best fit for usage of various purposes, we compared purity marker genes in Table 1 to assess the quality of isolated primary hepatocytes.

Reviewer #3:

Manuscript Summary:

The authors present a 2-step collagenase-based in vivo liver perfusion method for the isolation of primary mouse hepatocytes that is similar to many other published methods. The authors promote the efficiency of their method to isolate primary mouse hepatocytes that are suitable for conducting metabolism studies.

Major Concerns:

-The paper is lacking a number of important details that would need to be addressed in the video including: the required surgical equipment, means by which the mouse is secured for the procedure, mention of type of surface the mouse is adhered to, and steps taken to minimize bacterial contamination (e.g., conducting the procedure in a hood, preparing the mouse abdomen with an alcohol-based disinfectant, preparation of surgical instruments and other equipment prior to procedure).

Authors' reply: Thank you for your comment. We have included such information in manuscript: In Preparation section, "Sterilized forceps and scissors are prepared for dissection. To avoid possible contamination, it is also recommended to conduct all steps within sterile hood.", "Secure mouse on its back onto dissection pad by four limbs, by either pining or using water-proof tape or other methods approved by institution's Animal Care and Use Committee (or equivalents)." and in "Procedure" section, "Disinfect mouse abdomen with 70% EtOH.".

-Based on the provided data, the purity and survival of the isolated hepatocytes is questionable. The authors do not describe how survival was determined and the light microscopy images suggest both contamination and poor survival. Transcriptional and metabolic analyses of contaminated, dying, or dead hepatocytes may lead to erroneous conclusions.

Authors reply: Thank you for your comment. The live hepatocytes were purified using Percoll gradigent centrifugation. The survival rate was determined by Trypan blue staining, and we have added "measured with Trypan Blue staining according to manufacturer's protocol." in section "Discussion".

According to the activity analysis of pharmaceutical biomarkers and glucose metabolism, the isolated hepatocytes retained strong bioactivity for transcriptional and metabolic analysis.

-Maintaining the differentiated and in vivo metabolic state of primary mouse hepatocytes is very challenging. The authors should acknowledge the rapid decline in 'hepatocyte-like' gene expression. Here, is it important to note that the authors specifically discuss that the method does not use collagen coated plates or some type of attachment matrix. The authors may want to note that the use of these substrates is less to promote attachment as it is to promote and maintain hepatocyte function.

Authors reply: Thank you for your comment. We have included this information in manuscript in section of Representative Results: “After plating, their mRNA level decreased considerably with time, but levels still remained decent after 12 h, especially for ASGR1” in “Activity of pharmaceutical biomarkers was preserved”. Thank you also for the pointing out the usage of coating substrates is to promote and maintain hepatocyte function. Based on the purity marker gene tests, marker gene tests and metabolism tests in this study, the function of isolated hepatocytes should be maintained healthy.

Minor Concerns:

-Although temperature is highlighted as an important aspect of digestion, it does not appear that the solutions are not directly tested for temperature. It is recommended that a mock solution tube (of water) is stored under the same conditions as the coldest buffer and a thermometer is placed directly into that tub to ensure buffers at at the desired temperature.

Authors’ reply: Thank you for your comment and consideration. The mock solution tube should be of the same temperature as other tubes, as they are kept in same condition.

-No direct comparison against other protocols or collagenase-based buffers is made to demonstrate the claimed superiority of the method/commercial reagent.

Authors’ reply: Thank you for your comment. We have included the comparison of steps and method differences in discussion and Table 1. There is indeed no direct comparisons to other protocols. However, Figure 4-8 carefully tested the level of hepatocyte-related genes and activities, which showed comparable results with other similar protocols and proper functions, indicating the healthiness and activities of primary hepatocytes isolated with present protocol are of no considerable difference.

-The authors do not cite relatively recent similar methods published in JoVE.

https://www.jove.com/t/58323/isolation-primary-mouse-hepatocytes-for-nascent-protein-synthesis

https://www.jove.com/t/60507/isolation-3d-collagen-sandwich-culture-primary-mouse-hepatocytes-to

https://www.jove.com/v/56993/purification-hepatocytes-sinusoidal-endothelial-cells-from-mouse

Authors' reply: Thank you for your comment. We have cited and included the paper 58323 (Salem et al.) as reference 21, 60507 (Korelova et al.) as reference 23, and 56993 (Cabral et al.) as reference 22 in Table 1 for comparion.