July 6, 2018

Re: Manuscript ID JoVE58162

Dear Dr. DSouza:

Enclosed please find our revised manuscript entitled “An efficient sieving method to isolate intact glomeruli from adult rat kidney.”

We would like to thank you and the reviewers for their insightful and constructive comments. We have revised the manuscript by performing new experiments and revising the text. Detailed, point-by-point responses to the editor review and each individual reviewer are presented below. For your convenience, we have highlighted the changes in red font in the revision.

Your consideration is greatly appreciated.

Sincerely,

Roderick Tan, MD, PhD

Assistant Professor

Department of Medicine

University of Pittsburgh

**Editor Review comments:**

*• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.*

We have proofread our article in detail.

*•* ***Protocol Detail:*** *Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video.* ***Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) 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. Some examples:*

*1) 1.2.2.: Mention surgical tools used, incision sites etc. Please describe all surgery steps.  
2) 1.3.1: using scalpel?  
3) 2.4, 3.1: mention centrifuge speed (in g), duration and temperature.*

Thank you for pointing these out, we have included the details requested.

*•* ***Protocol Numbering:*** *Please adjust the numbering of your protocol section to follow JoVE’s instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.*

We have numbered the protocol steps according to JoVE instructions.

*•* ***Protocol Highlight:*** *After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.*

*1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.  
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.  
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.  
4) Notes cannot be filmed and should be excluded from highlighting.*

Since we have exceeded 3 pages, we have highlighted the most relevant section of the protocol for this manuscript.

*•* ***Discussion:*** *JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.*

We believe that our discussion is greatly improved and we tried to focus on the protocol and any shortcomings.

*•* ***Figures:*** *1) Please remove the text “Figure #..” from the figure files.  
2) Fig 1B, C, 2 A-C, #A,B, : Please add scale bars.  
3) Fig 3 E,F: Please discuss these figures in your results section. Please also use the original size blots. Are these all on the same gel?*

We have removed the word Figure from each figure file. For the scale bars, we believe that addition of scale bars would clutter each image and necessitate increasing the size of each image to include the bar. Since Fig. 1A and its scale bar clearly shows the size of each glomerulus we believe that additional scale bars would be redundant. However, we will add them, if it is believed necessary in spite of this explanation. We have included reference to Fig 3E-F in our text. The westerns were from the same gel.

*•* ***Commercial Language:****JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Poly/Bed® 812 (Luft),  
1) Please use MS Word’s find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.*

We removed the poly/bed 812 (Luft) commercial language.

*• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."*

We do not use any previously published images.

**Responses to reviewers’ comments:**

**Reviewer #1:**

*The fundamental weakness of the protocol "An Efficient Sieving Method to Isolate Glomeruli from Adult Rats for Primary Culture" is the almost complete absence of scholarship concerning both previously published methods for glomerular isolation by sieving and the problems inherent in using sieved glomeruli in subsequent experiments. Glomerular isolation by sieving in combination with perfusion of iron particles and magnetic purification was first described in 1958 (Nature 182:1103-4), and a subsequent publication in 1972 described a procedure, very similar to the method described in the protocol under review, which used only sieving and centrifugation (Am J Clin Path 58:135-9). Multiple refinements of glomerular sieving protocols have appeared in the literature since then, but neither the base references nor the later work were cited by Rush et al., leaving the false impression that their protocol is the only alternative to glomerular isolation procedures using infusion of ferrous particles and magnetic purification (such as the single isolation protocol cited).*

We agree with the reviewers’ concerns and have now included a comprehensive assessment of the literature including the landmark papers listed above. We have also included a discussion of the magnetic preparations that have also been used to isolate glomeruli.

*The authors also make a point of touting the value of their protocol as a source of purified glomeruli for subsequent experiments and primary culture. There is considerable literature concerning the viability of cultured glomeruli and their proclivity for undergoing rapid apoptosis (e.g., Kidney Int 54:2008-13), the pitfalls of studies using sieved glomeruli (e.g., Nephrol Dial Transplant 20:3055-60), use of glomerular outgrowths as a source of primary cultures (e.g., Kidney Blood Press Res 30:162-74), as well as how sieved glomeruli can be used to measure the permeability of the glomerular filtration barrier (e.g. J Am Soc Nephrol 9:433-438, and, more recently, an article in press at Kidney International at* [*http://www.kidney-international.theisn.org/article/S0085-2538(17)30895-5/pdf)*](http://www.kidney-international.theisn.org/article/S0085-2538(17)30895-5/pdf))*. None of this work was cited in the current manuscript and the authors indeed seemed unaware of it, as they wrote that, ". . . we were unable to design a functional assay in which permeabiliity could be tested." despite the >20 year history of permeability assays using sieved glomeruli described in published papers.* *In the reviewer's personal experience, glomeruli isolated by sieving undergo apoptosis quite rapidly, and this should be added as a caveat to the protocol paper. In addition, though treatment of cultured podocytes with protamine sulfate at 600 µg/ml has been published, the reviewer has found (though not published) that protamine sulfate at this concentration rapidly kills cultured podocytes in <20 min, and is very likely to rapidly kill other glomerular cells. The authors need to show the results of analyses of cell viability and of cells undergoing apoptosis for both sieved glomeruli and "injured" (by protamine sulfate) glomeruli in order to accurately assess the utility of their protocols to produce culturable glomeruli and to be useful as a model of glomerular injury.*

We thank the reviewer for these comments. It is indeed true that apoptosis of glomerular cells occurs after isolation. To assess viability we performed new experiments in which we assessed isolated glomeruli for cleaved caspase-3 (by immunofluorescence) in order to identify cells undergoing apoptosis. As shown in the new figure 4, cleaved caspase-3 appears in isolated glomeruli 2 hours after isolation, with levels accumulating progressively through 48 hours. This suggests that downstream applications should be performed immediately after isolation, in order to minimize effects of apoptosis on these experiments.

We also agree that there are pitfalls to this isolation protocol and have enhanced our discussion of these in our manuscript. We have especially focused on the outgrowth of cells from the glomeruli which is an area of controversy, with some investigators identifying them as podocytes, some as parietal epithelial cells, and sometimes as both. We have also discussed the methodology papers describing assessment of glomerular permeability by volume or by immunofluorescence.

*Nephrin labeling should be adequate to identify glomerular podocytes, since WT-1 labels podocyte nuclei, which doesn't add much to the IF labeling shown in Figures 2 & 3. The lack of WT-1 and nephrin labeling in the protamine sulfate "injured" glomeruli suggests to me that all the cells in treated glomeruli were dead and their nuclei lysed, not that the podocytes were "injured," except insofar as fatal injury can be called injury after death occurs.*

We elected to keep the nephrin labeling to reassure readers that this slit diaphragm protein remained intact in our preparations. We agree it is possible that protamine sulfate-induced loss of WT1 and nephrin staining might indicate cell death but only used protamine sulfate to show that experimental manipulations could reduce nephrin staining and cause podocyte foot process effacement, which we believe is shown in our data.

*The protocol itself is rather rambling and needs clarification and tightening up. For example, in Section 1.3.1:*We thank the reviewer for the close review of our protocol. We have made extensive revisions and believe that the protocol is now easier to understand.

**Reviewer #2:**

*This particular group is just re-hashing a series of protocols that was first developed by Meezan and Carlson in 1975 for isolation of glomerular basement membrane but was later refined/modified to culture glomeruli and glomerular cells by Jordan Kreisburg and Morris Karnovsky in 1978. I am sort of surprised there is no mention of their papers in this particular manuscript. Those of us in the kidney field are all familiar with their work and usually refer to those protocols in the methods section of our own manuscripts. The protocol itself provides no significant advance with regard to glomerular isolation. Again most of us who work in the kidney field use the serial sieving protocol on a routine basis and achieve a similar efficiency of glomerular recovery. As mentioned above the protocol is well worked out in the literature.*  
  
We thank the reviewer for these comments on our manuscript. We agree that we should include and discuss references to the original studies for this procedure. While our protocol does not markedly differ from prior studies we believe it is a useful procedure to publish in an open-access manner for the scientific community.

*The TEM micrographs are out of focus (especially Panel D) and overall the TEM fixation is not very well done.*

We have taken new photographs to improve our figures. We do address that some fixation artifacts are noted with TEM in our protocol.   
  
  
**Reviewer #3:**   
  
*1. The method described in present study has already been utilized in many previous reports, for example [Biochim Biophys Acta. 2015 Aug;1852(8):1599-609.], however, the diameters of three consecutive sieves are different, so the novelty and difference from previous studies should be described clearly.*

We thank the reviewer for this comment. We have included references to the original and subsequent work to place our protocol in context. A variety of sieve sizes have been used over the years with similar overall results.

*2. According to Figure 1, the glomeruli isolated in this study are decapsulated, the authors should clarify in the paper.*

Thank you for this comment. It is true that encapsulated and decapsulated glomeruli may behave differently and would lead to different cellular outgrowths from the glomeruli. We address these in our discussion section.

*3. There are several typing mistakes in the manuscript, for example "Nephin" and "um" in the TABLE OF MATERIALS/EQUIPMENT section. The authors should go through the manuscript carefully.*

We have corrected the typographic errors throughout.