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## An efficient sieving method to isolate intact glomeruli from adult rat kidney

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**TITLE:**

An Efficient Sieving Method to Isolate Intact Glomeruli from Adult Rat Kidney

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Kidney, Foot Process Effacement, Glomerular Injury, Proteinuria, Glomerular Permeability, Protamine Sulfate, Renal Pathology, Glomerular Disease

**SHORT ABSTRACT:**

The main focus of this protocol is to efficiently isolate viable primary glomeruli cultures with minimal contaminants for use in a variety of downstream applications. The isolated glomeruli retain structural relationships between component cell types and can be cultured *ex vivo* for a short time.

**LONG ABSTRACT:**

Preservation of glomerular structure and function is pivotal in the prevention of glomerulonephritis, a category of kidney disease characterized by proteinuria which can eventually lead to chronic and end-stage renal disease. The glomerulus is a complex apparatus responsible for the filtration of plasma from the body. In disease, structural integrity is lost and allows for the abnormal leakage of plasma contents into the urine. A method to isolate and examine glomeruli in culture is critical for the study of these diseases. In this protocol, an efficient method of retrieving intact glomeruli from adult rat kidneys while conserving structural and morphological characteristics is described. This process is capable of generating high yields of glomeruli per kidney with minimal contamination from other nephron segments. With these glomeruli, injury conditions can be mimicked by incubating them with a variety of chemical toxins, including protamine sulfate, which causes foot process effacement and proteinuria in animal models. Degree of injury can be assessed using transmission electron microscopy, immunofluorescence staining, and western blotting. Nephron and Wilms Tumor-1 (WT-1) levels

can also be assessed from these cultures. Due to the ease and flexibility of this protocol, the isolated glomeruli can be utilized as described or in a way that best suits the needs of the researcher to help better study glomerular health and structure in diseased states.

## INTRODUCTION:

The glomerulus is a highly specialized tuft of capillaries responsible for the filtration of circulating plasma. It forms the beginning of the nephron, which is the basic functional unit of the kidney. Glomerular function is defined by a uniquely fenestrated capillary endothelium, the slit diaphragm of podocytes, and an intervening basement membrane. These layers form a semipermeable barrier to allow for the selective excretion of substances into the filtrate. Water, ions, and other small molecules generally pass through, while larger molecules are retained in the plasma. Podocytes are specialized epithelial cells that spread over the basement membrane, surrounding the capillaries with cytoplasmic projections known as foot processes. The foot processes of adjacent podocytes interdigitate and are crossed by slit diaphragms comprised of proteins such as nephrin, podocin, P-cadherin, and ZO-1<sup>1</sup>. In cross section, these foot processes are evenly arranged over the basement membrane. In diseased glomeruli, the foot processes become grossly abnormal or “effaced,” leading to abnormal leak of plasma contents into the filtrate. As such, glomerular damage is generally characterized by the presence of abnormally large amounts of protein (*e.g.*, proteinuria) and/or red blood cells (*e.g.*, hematuria) in the urine. In addition, injured podocytes lose expression of nephrin as well as its regulator Wilms Tumor-1 (WT-1), a key protein responsible for maintenance of differentiation<sup>2,3</sup>. The glomeruli are a primary target of damage in diabetic nephropathy and other glomerulonephritides such as minimal change disease, membranous nephropathy, and focal segmental glomerulosclerosis. These diseases are major causes of progressive kidney failure and the development of end-stage renal disease, a condition in which survival relies upon dialysis or renal transplantation. Therefore, it is important to study glomeruli to better understand chronic kidney disease (CKD) pathology.

A cell culture system is critical to studying glomerular biology. Due to its central role in generating the slit diaphragm, as well as the existence of specific proteinuric diseases due to slit diaphragm protein mutations, much research has understandably utilized the podocyte in isolation. This has led to the generation of primary podocyte cell lines to utilize *in vitro*. These cells can be cultured under a variety of conditions and can even be grown on permeable supports to assess permeability<sup>4</sup>. However, the isolation of proliferating cells often selects dedifferentiated cells causes that have lost of some podocyte markers. This has led to the generation of conditionally immortalized podocytes derived from a transgenic mouse strain carrying a temperature-sensitive mutant of the SV40 large T gene (*e.g.* immortalmouse), which could be grown in culture but also be differentiated to express a full array of podocyte markers<sup>5</sup>. These methods of primary culture have been pivotal in understanding podocyte biology<sup>4,6,7</sup>.

Nevertheless, cultures containing single cell types lack the intercellular relationships that occur *in vivo* as well as the support structure and matrices, and monolayers of these cells do not necessarily recapitulate the three-dimensional architecture of glomeruli. The immortalized podocytes can also be cumbersome and challenging to culture<sup>8</sup>, and require possession of either

the immortalized mouse or a starting aliquot of cells from established investigators to get started. Further, the glomerulus is comprised of not only podocytes, but also capillary endothelial cells and the basement membrane, as well as mesangial cells which provide support for the structure. It is therefore useful to develop an *ex vivo* approach available to all investigators for the study of intact glomeruli that retain their native architecture as well as all the cells constituting the normal glomerulus.

In 1958, Cook and Pickering described the first isolation of glomeruli from the rabbit kidney. After observations that fat emboli became lodged in glomeruli, they postulated that particles of the same size could be used to specifically isolate these structures. Indeed, the infusion of iron oxide particles into the kidney led to the trapping of these particles in glomeruli. After mechanical dissociation and sieving of the kidney, the glomeruli could be isolated intact and with purity through the use of magnetic separation<sup>9</sup>. In 1971, Misra showed that the iron oxide infusions could be omitted, and glomerular isolation achieved with sieving of minced human, dog, rabbit or rat kidney tissue<sup>10</sup>. This technique has been modified since then depending on the goal of the investigators but has essentially resulted in purified preparations that could be further studied or from which primary cell cultures could be established<sup>11-17</sup>.

Here we describe a protocol for the isolation of intact viable glomeruli from the rat kidney. The entire protocol takes just a few hours. Although they do not proliferate, experimental plans of any size can be supported by simply increasing the number of kidneys as starting material. While there are published protocols for the magnetic bead separation of glomeruli, they require an intravenous injection of beads, are more expensive, and may alter biology since the beads are either retained by the glomeruli in culture or require glomerular “lysing” and removal by centrifugation<sup>19</sup>. Compared to mouse glomeruli, the larger size of rat glomeruli (nearly 100  $\mu$ m in two-month old rats<sup>18</sup>) makes it much easier to separate them from other kidney structures using a simple sieving technique.

As evidence of their usefulness, we have characterized the glomeruli to demonstrate the different cell types. They can also be exposed to agents known to injure glomeruli *in vivo*, and we demonstrate the adverse effects of protamine sulfate (PS) on these cultures. PS is a polycation that neutralizes the anionic sites along the glomerular capillary wall<sup>20</sup>. This neutralization has a dramatic effect on the glomerular filtration barrier and therefore increases proteinuria and foot process effacement. These glomeruli can be assessed with immunoblots for key proteins such as nephrin and WT-1 to assess overall health. Furthermore, their structure can be evaluated with light, immunofluorescence, and electron microscopy.

Overall, this protocol is accessible to most investigators (one only needs access to the animals and some simple equipment). With morphological features left undamaged, the researcher is able to analyze the glomeruli and see how other important cell types and matrix preservation in the glomeruli affect function and disease progression, a shortcoming of podocyte cultures.

## **PROTOCOL:**

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of University of Pittsburgh.

## 1. Isolation of Rat Glomeruli

1.1. To prepare a sterile 1% isolation buffer, add 5 g of bovine serum albumin (BSA) to a 600 mL beaker.

1.1.1. Add 500 mL of 1x phosphate buffered saline (PBS) to the beaker and stir with a magnetic stirring rod until all BSA is dissolved.

1.1.2. Sterile filter the 1% BSA/PBS buffer in a cell culture hood.

Note: The sterile 1% BSA/PBS buffer can be kept at 4 °C for up to a week.

1.2. Obtain 2 to 4 Sprague-Dawley rats weighing 150 to 200 g each.

1.2.1. Euthanize rats *via* carbon dioxide inhalation using a chamber in which 100% carbon dioxide is introduced at a rate of 10-30% of the chamber volume per minute. Observe animals for cessation of respiration and faded eye color before removal from carbon dioxide.

1.2.2. Prepare the skin over the anterior abdomen with 70% ethanol and utilize hair clippers to remove hair, if desired. Make a midline incision in the skin using surgical scissors or scalpel. Make another midline incision through the muscle layer to expose internal organs. Extend the incision superiorly through the diaphragm and sternum or rib cage as a secondary method of euthanasia.

1.2.3. Isolate and remove both kidneys and place into a sterile 50 mL plastic conical tube with 30 mL of Hanks' buffered salt solution (HBSS) on ice.

Note: Several rats can be euthanized in the same chamber and all of the kidneys can be placed in the same conical tube.

1.3. Keep the kidneys on ice and transport to a sterile cell culture hood. Transfer the kidneys to a sterile Petri dish containing 5 mL of HBSS, also over ice, and remove and discard perirenal fat with scissors and sharp forceps. If still present, also remove and discard the capsule surrounding the kidney by making a small superficial incision and then use sharp forceps to gently pull it away from the organ.

Note: Always keep kidney isolates on ice and practice sterile technique throughout. A piece of sterile gauze can be placed in the Petri dish as a textured surface to hold the kidney in place during manipulation.

1.3.1. Cut kidneys in half lengthwise (midsagittal section) and remove and discard the medulla (which is darker in color) with a scalpel in a second Petri dish with 5 mL of HBSS.

176  
177 1.3.2. Transfer the remaining pieces, which are predominantly kidney cortex, into a third Petri  
178 dish with 5 mL of HBSS and mince with a sterile razor blade until the pieces are less than 1 mm  
179 in size, or until a paste is formed.

180  
181 1.4. Wet the top and bottom of a 180  $\mu$ m sieve with 1% BSA/PBS over a 500 mL waste beaker.  
182 This step is critical as it coats the sieve with protein and reduces adherence of glomeruli, which  
183 will improve yield.

184  
185 1.5. Place the minced cortex on a small edge of the sieve and use the textured plunger flange  
186 (the side opposite the rubber seal) of a 10 mL syringe to mush the tissue through the sieve into  
187 a bottom pan sitting on ice. Rinse periodically with HBSS but use as little as possible to avoid  
188 sample dilution.

189  
190 Note: Do not exceed 30 mL of buffer total, so only 25 mL more can be used here. The reason for  
191 placing the minced tissue on only one edge of the sieve is to reduce adherence of glomeruli by  
192 limiting exposure to part of the sieve rather than the entire sieve surface area.

193  
194 1.5.1. To facilitate this, reuse the fluid collecting in the bottom pan to rinse the sieve. Once sieving  
195 is complete, carefully wash the bottom of the sieve once more with 1% BSA/PBS buffer from the  
196 bottom of the pan to capture any glomeruli that may be loosely adhered.

197  
198 1.5.2. Collect all of the fluid in the bottom pan into several 10 mL syringes equipped with 20 G  
199 needles and pass it through the needle at least 2 additional times. On the last collection, keep  
200 the glomeruli-containing fluid in the syringe until ready to pass it through the 90  $\mu$ m sieve.

201  
202 1.6. While the fluid is stored in the syringes, wash the bottom pan by flushing with 1% BSA/PBS  
203 into waste beaker and wet the top and bottom of a 90  $\mu$ m sieve with 1% BSA/PBS. Place the sieve  
204 on top of the bottom pan on ice.

205  
206 1.6.1. Apply the sample in the syringes to one edge of the sieve and mush through the sieve with  
207 another syringe flange as describe above. Wash with solution from the bottom pan to collect  
208 everything on one edge of the sieve.

209  
210 1.6.2 Once sieving is complete, carefully wash the bottom of the sieve as in step 1.5.1. Collect all  
211 the fluid in the bottom pan in a 50 mL plastic conical tube.

212  
213 1.7. Wash the bottom pan with 1% BSA/PBS into a waste beaker and wet the top and bottom of  
214 a 75  $\mu$ m sieve with 1% BSA/PBS. Place the sieve on top of the bottom pan on ice.

215  
216 1.7.1. Apply the sample to one edge of the sieve. Liquid should flow through easily. Rinse through  
217 the top with at least 20 mL of 1% BSA/PBS into waste beaker. Carefully wash the bottom of the  
218 sieve as well. The glomeruli will remain on the top of this 75  $\mu$ m sieve.

219

1.7.2. Use HBSS to collect glomeruli in a Petri dish by rinsing through the sieve upside down. Use as much HBSS as needed here. Collect into one or more 50 mL plastic conical tube(s) on ice.

1.8. Centrifuge at 1800 x g for 5 min at 4 °C, remove supernatant carefully with a pipette, and resuspend pellet in 10 mL of cold HBSS. Combine the samples if multiple conical tubes were collected in 1.7.2. Repeat the centrifugation step.

1.9. Resuspend the glomeruli in 5 mL of HBSS until ready to proceed to the next step.

1.10. If desired, take a count of the total glomeruli. For this, take a 10 µL sample with a pipette and place the drop on a glass slide. View under a microscope, count the glomeruli in the field and multiply by 500 to get total yield.

Note: Purity may be determined by counting the number of tubules seen in the field and dividing by total number of glomeruli and tubule structures. There should be > 95% glomeruli in the visual field.

1.11. If there is significant tubular contamination, repeat steps 1.6.1 onward, and make sure to wash thoroughly when completing step 1.7.1. This is the step in which tubular contamination is most likely to occur.

## **2. Injury of Glomeruli**

2.1. Collect the glomeruli in a 15 mL plastic conical tube and spin down at 200 x g. Resuspend in an appropriate amount of HBSS based on the total yield so that there are approximately 9,000 glomeruli per well. Pipette 450 µL of sample into each well of a 24-well plate, or any plate format that fits the downstream assays required.

Note: Pipetting up and down to mix glomeruli in buffer ensures a homogeneous solution. The glomeruli are very sticky and heavy and will stick to each other as well as settle at the bottom of a solution quickly and onto the sides of the tube.

2.2. To make 6 mg/mL protamine sulfate (PS) solution, first dissolve 1 g of PS into 10 mL of dH<sub>2</sub>O heated to 40 °C in a 15 mL plastic conical tube and vortex thoroughly. Take 30 µL of the solution and add to 470 µL of dH<sub>2</sub>O.

Note: This will produce a 6 mg/mL solution, and when 50 µL are added to the well as described below, the final concentration is 0.6 mg/mL.

2.2.1. In duplicate, add 50 µL of protamine sulfate (this is a 1:10 dilution) to each well of one group, while supplying another group 50 µL of HBSS (negative control).

2.3. Incubate the plate for 4 h at 37 °C.

264  
265 2.4. Spin down contents of each well in a microcentrifuge tube (4000 x g, 4 °C, 10 - 15 s) and wash  
266 twice with 1 mL of PBS each.

267  
268 2.5. Aspirate off the final wash and resuspend in 300 µL of PBS. Split into three aliquots to be  
269 prepared for protein isolation, transmission electron microscope (TEM) visualization, and  
270 immunofluorescence staining (IF).

### 271 272 **3. Preparing Glomeruli for Analysis**

273  
274 3.1. Spin down the contents of the three aliquots (4000 x g, 4 °C, 10 - 15 s) and aspirate off  
275 remaining buffer and proceed to sample analysis by TEM, IF, or protein isolation.

#### 276 277 3.2. Processing for TEM

278  
279 3.2.1. Fix glomerular pellets in 150 µL of cold 2.5% glutaraldehyde in 0.01 M PBS. Remove the  
280 fixative carefully from the pellet using a Pasteur pipette, making sure not to disrupt the pellet,  
281 then rinsed it with PBS and post-fix it in 1% osmium tetroxide with 1% potassium ferricyanide.

282  
283 3.2.2. Dehydrate the sample through a graded series of ethanol (30, 50, 70, 90, 100, 100, 100%)  
284 and propylene oxide then embed in epoxy embedding material.

285  
286 3.2.3. Cut semi-thin (300 nm) sections on an ultramicrotome. Stain with 0.5% Toluidine Blue in  
287 1% sodium borate and examine them under the light microscope.

288  
289 3.2.4. Cut ultrathin sections (65 nm), stain them with uranyl acetate and Reynold's lead citrate,  
290 and examine on a transmission electron microscope.

#### 291 292 3.3. Processing for IF

293  
294 3.3.1. Add 150 µL of a 12% gelatin in PBS solution and immediately place on dry ice to form a  
295 pellet. Soak the pellet in 500 µL of 2% paraformaldehyde/1x PBS in a microcentrifuge tube  
296 overnight at 4 °C.

297  
298 3.3.2. Place the pellet in a base mold and embed by adding optimal cutting temperature (OCT)  
299 medium over dry ice. Cut 5-10 µm sections on a cryotome and proceed with  
300 immunofluorescence/immunohistochemistry or standard histological stains.

#### 301 302 3.4. For Protein Isolation

303  
304 3.4.1. Add 150 µL of RIPA buffer to centrifuged glomerular pellet with 1.5 µL of protease inhibitor  
305 and dounce with tissue homogenizers.

306  
307 3.4.2. Proceed to protein quantification and immunoblotting or other protein analysis.



## REPRESENTATIVE RESULTS:

The protocol from the time of euthanasia to isolation of the glomeruli can be completed in as little as 2 h and has a high throughput and efficiency. With proper utilization of the technique, the yield of glomeruli per rat kidney ranges from 6,000 - 10,000 glomeruli when starting with 8 kidneys. The final suspension is densely packed with glomeruli and has an overall purity > 95%, showing minimal contamination from tubular segments or other cell types (**Figure 1A, B**). In addition, the OCT-embedded glomeruli can be stained with Hematoxylin and Eosin (H & E) stains to see the morphology (**Figure 1C**). These glomeruli maintain their structure throughout the whole protocol, even after processing. We demonstrate that isolated glomeruli possess intact and viable podocytes (**Figure 2A**), mesangial cells (**Figure 2B**), and endothelial cells (**Figure 2C**).

Once isolated, the glomeruli can be exposed to well-known chemical injuries to simulate *in vivo* pathology. In this case, protamine sulfate (PS) was chosen for its ability to disrupt the charge of the glomerular filtration barrier, which eventually leads to foot process effacement. PS-treated glomeruli have a striking reduction in nephrin (red) and a number of nuclei positive for WT-1 (green) *via* immunofluorescence (**Figure 3A, B**). The glomeruli can also be prepared for transmission electron microscopy (**Figure 3C**). Control samples have normal podocyte morphology and foot processes whereas the PS-treated glomeruli have foot process effacement (**Figure 3D**), which is a sign of podocyte dysfunction and is seen in *in vivo* models using PS<sup>21</sup>. This also corresponded to a decrease in nephrin detected by immunoblotting (**Figure 3E, F**).

To determine viability, cleaved caspase-3 was assessed as a marker of apoptosis. Using immunofluorescence, cleaved caspase-3 first appears in a few cells starting 2 h after isolation (**Figure 4**). The number of cells expressing this protease became more abundant over time, with the highest levels seen at 24 and 48 h. This suggests that apoptosis does occur relatively early in culture and that downstream applications should be performed soon after isolation for best results.

## FIGURE LEGENDS:

**Figure 1. Typical appearance of rat glomerular cultures after isolation.** (A) Brightfield image of glomerular culture. Although we chose a field in which there is a single renal tubule in this micrograph (arrowhead), the cultures obtained are generally > 95% pure. Scale bar equals 100  $\mu$ m. (B) Enlarged image of a single glomerulus. (C) Hematoxylin and eosin stain of a single glomerulus. Scale bars in B and C equal 10  $\mu$ m.

**Figure 2. Constituent cell types are retained in isolated glomeruli.** Confocal immunofluorescence microscopy was performed for nephrin (red, podocytes) and cell-specific markers to identify podocytes, mesangial cells, and the endothelium. (A) Costain for nephrin and WT1 (green, podocytes). (B) Costain for nephrin and PDGFR- $\beta$  (green, mesangial cells, arrow). (C) Costain for nephrin and CD31 (green, endothelial cells, vessels in cross section marked by arrowheads). Scale bar = 25  $\mu$ m on all panels.

**Figure 3. Podocyte injury can be induced *in vitro* using isolated rat glomeruli.** (A) Confocal immunofluorescence microscopy for nephrin and WT1 in an untreated glomerular culture. Note the linear nephrin staining (red) and presence of WT1-positive nuclei (green) which are typically seen when there are healthy podocytes. (B) After protamine sulfate treatment, nephrin expression is decreased and non-linear, and WT1 is absent, indicating podocyte injury. (C) Transmission electron microscopy (TEM) image showing foot processes, basement membrane, and a fenestrated endothelium typical of normal glomerular structure. Bar equals 500 nm. (D) After protamine sulfate, foot processes are elongated or effaced (arrowheads), which indicates podocyte injury. (E) Western blot for nephrin showing decreased nephrin after protamine sulfate treatment. (F) Actin loading control for western blot is shown.

**Figure 4. Assessment of cell viability in isolated glomeruli.** Confocal immunofluorescence microscopy for cleaved caspase-3 (green) was performed. A nephrin co-stain was performed to easily locate the glomeruli. While there was no cleaved caspase-3 positivity at 0 and 1 h after isolation of glomeruli, fluorescence signal in a few cells was noted at 2 h. Progressively more cells turned positive the longer after isolation they were examined. The greatest number of caspase-3 positive cells were noted at 24 and 48 h.

#### **DISCUSSION:**

This is an efficient method for recovering glomeruli from rat kidney using inexpensive, reusable equipment with a simple protocol. As with all procedures, there are limitations to its usefulness. First, although we obtain > 95% purity, because of the sieving nature of the protocol and the starting material it is impossible to exclude all contaminants, and a few red blood cells and the occasional tubular segment will be present in the culture. We do not anticipate these small contaminants to be a problem for the vast majority of applications. Second, the sieving protocol relies on the use of rat kidneys, in which glomeruli are much larger than in mice. If mouse glomeruli are needed, a technique using commercial magnetic beads (*e.g.*, Dynabeads) has been published<sup>22</sup>. Third, it has been shown that specific mRNAs (plasminogen activator inhibitor-1 and collagen I) in isolated glomeruli derive almost entirely from Bowman's capsule rather than intraglomerular cells<sup>23</sup>. This could lead to inappropriate conclusions if mRNA isolation is attempted from these glomeruli. It should be noted that in our hands the majority of glomeruli are decapsulated and should therefore lack significant contributions from Bowman's capsules. Fourth, cell death begins to occur relatively quickly under culture conditions.

We found that the apoptotic program, as evidenced by appearance of cleaved caspase-3, was activated starting at 2 h of culture. This is in general agreement with previous reports showing that apoptosis, as assessed by DNA fragmentation, TUNEL, and histologic analysis, begins to occur within 1-2 h after isolation<sup>24</sup>. However, it should also be noted that early observations showed that metabolic activity could be detected from isolated sieved glomeruli when cultured for at least 3 h, suggesting considerable cellular viability at this timepoint<sup>16</sup>. Nevertheless, based on the results, we believe that it would be prudent to utilize isolated glomeruli immediately in intended experiments for the best results. We recommend that all experiments be performed before 72 h as we have observed that the entire glomerular structure begins to deteriorate beyond that timepoint.

It is much easier to obtain higher yields when starting with 4-8 kidneys as opposed to only 2 because a certain number of glomeruli are lost due to adherence to the various sieves, but once the sieves are maximally coated there is no additional loss. For the same reason, it is important to soak the sieves in the BSA/PBS buffer prior to use as glomeruli are more likely to adhere to a dry sieve, and to limit tissue exposure to one edge of the sieve. We suggest starting with no fewer than 6 kidneys (3 rats) to obtain a reasonable yield.

Some investigators have isolated primary podocyte cultures from isolated glomeruli which tend to grow out of the glomeruli during culture<sup>17</sup>. We have noted that some isolated glomeruli will adhere to the culture dish plastic and that cells will begin to migrate out of the glomerular structure at late timepoints (72 h after isolation). The study of these cells is beyond the scope of this isolation protocol, and there is some controversy as to whether these cells are podocytes, parietal epithelial cells, or both<sup>15,25</sup>. Notably, Mundel *et al.*, have reported that cobblestone cells harvested from sieved glomeruli may be induced to differentiate into mature podocytes under specific culturing conditions<sup>26</sup>. Some of the confusion regarding cell identity may depend on whether the isolated glomeruli are encapsulated (including Bowman's capsule which is populated by parietal epithelial cells) or decapsulated in the sieving procedure. In our hands, using sequential sieve sizes of 180, 90, and 75  $\mu\text{m}$  led to the majority of glomeruli being decapsulated.

Most forms of proteinuric chronic kidney disease are due to increases in glomerular permeability. Several authors have utilized isolated glomeruli in *ex vivo* permeability experiments. In one method, the change in glomerular volume after altering the osmotic content of the surrounding media was used to estimate permeability<sup>27</sup>. Recently, it was established that leakage of a fluorescent probe from isolated glomeruli could be quantified to measure permeability and could be performed in rodents exposed to glomerular disease experimental models<sup>28</sup>.

We have noted that in the TEM preparation process, we sometimes see the podocyte foot processes lift off the basement membrane. We do not feel this is happening in the culture and is more likely an artifact during TEM sample preparation. Notably, even when this occurs, it is clear whether the foot processes look "normal" or are effaced.

Overall, this protocol provides a method by which one can evaluate morphologic and cellular changes in response to injury. We anticipate that it may be used as a companion technique to isolated podocyte cultures, especially when interactions with extracellular matrix or other native cell types are being considered. It holds promise for increasing understanding of proteinuric CKD, which would improve the ability to develop future therapeutics for this debilitating disease.

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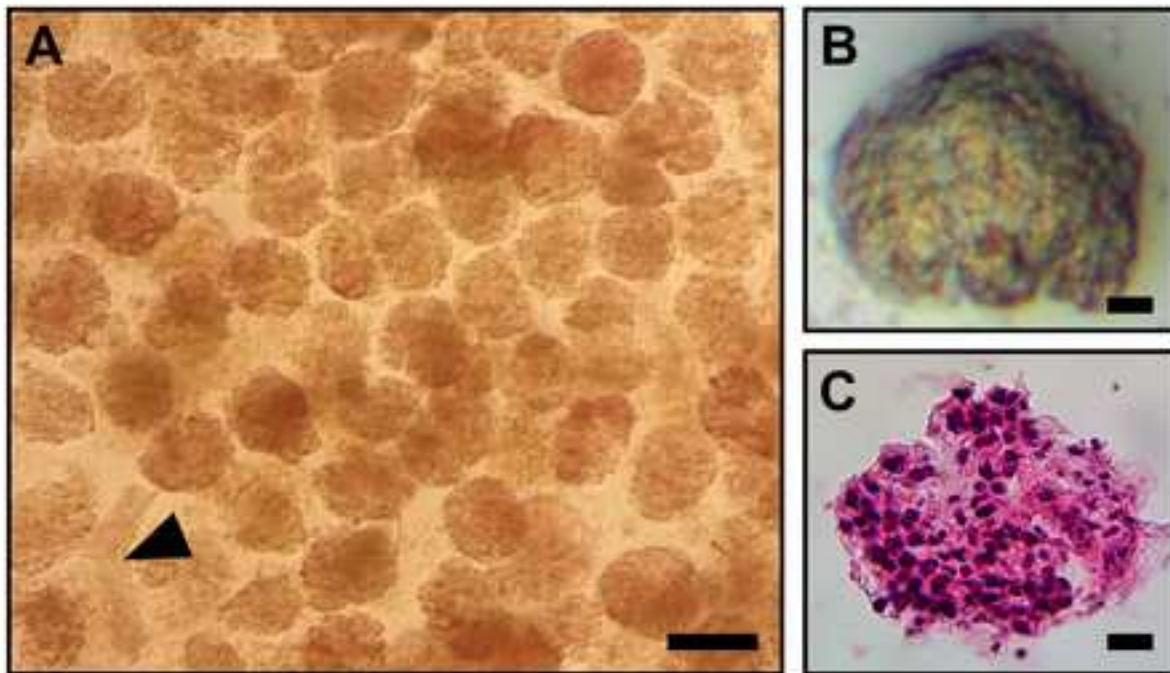
#### DISCLOSURES:

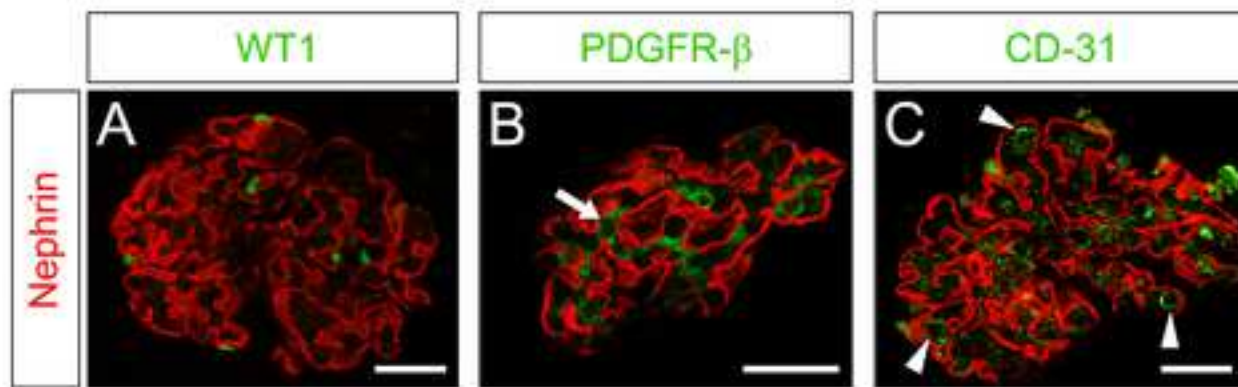
The authors have nothing to disclose.

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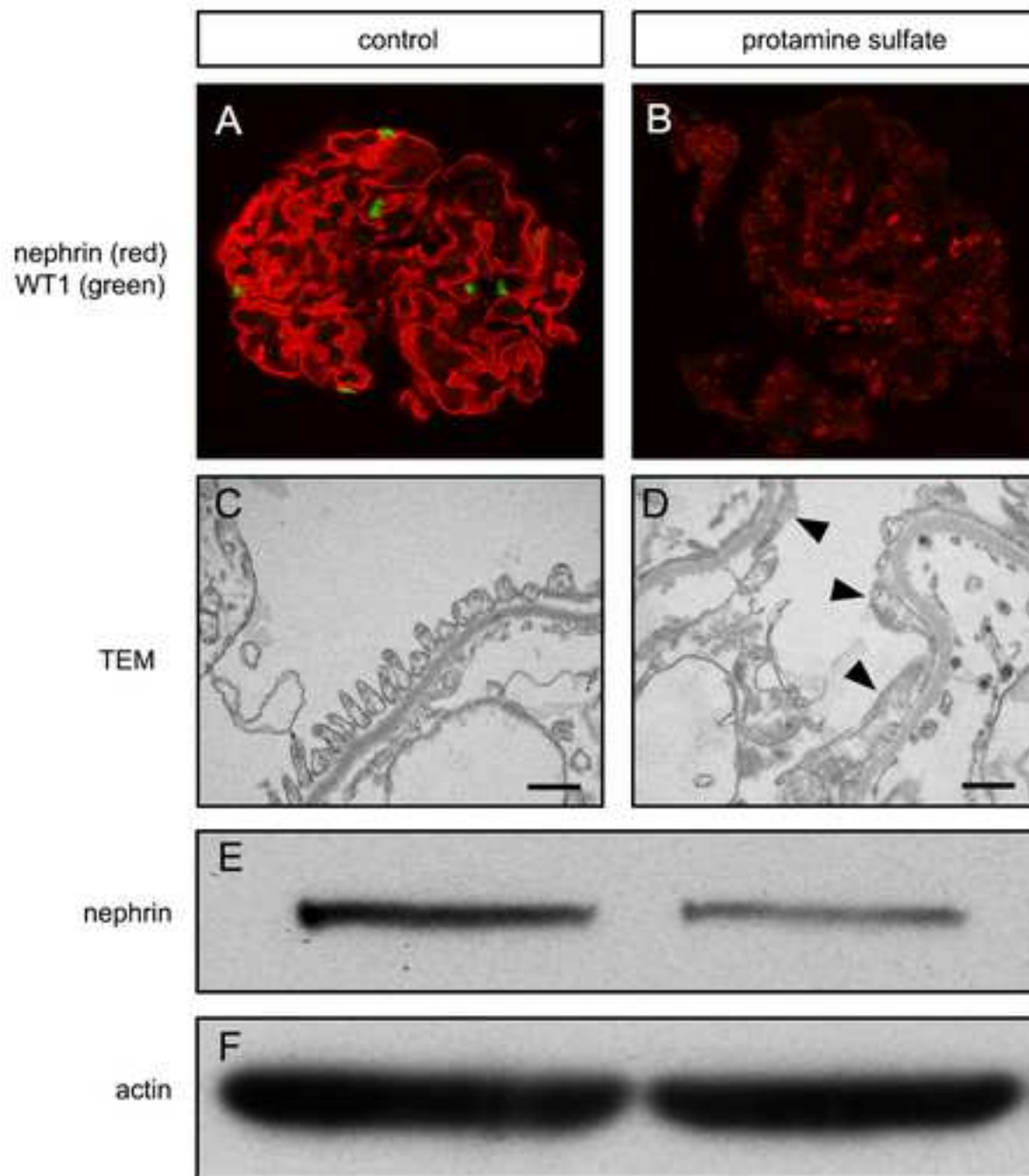
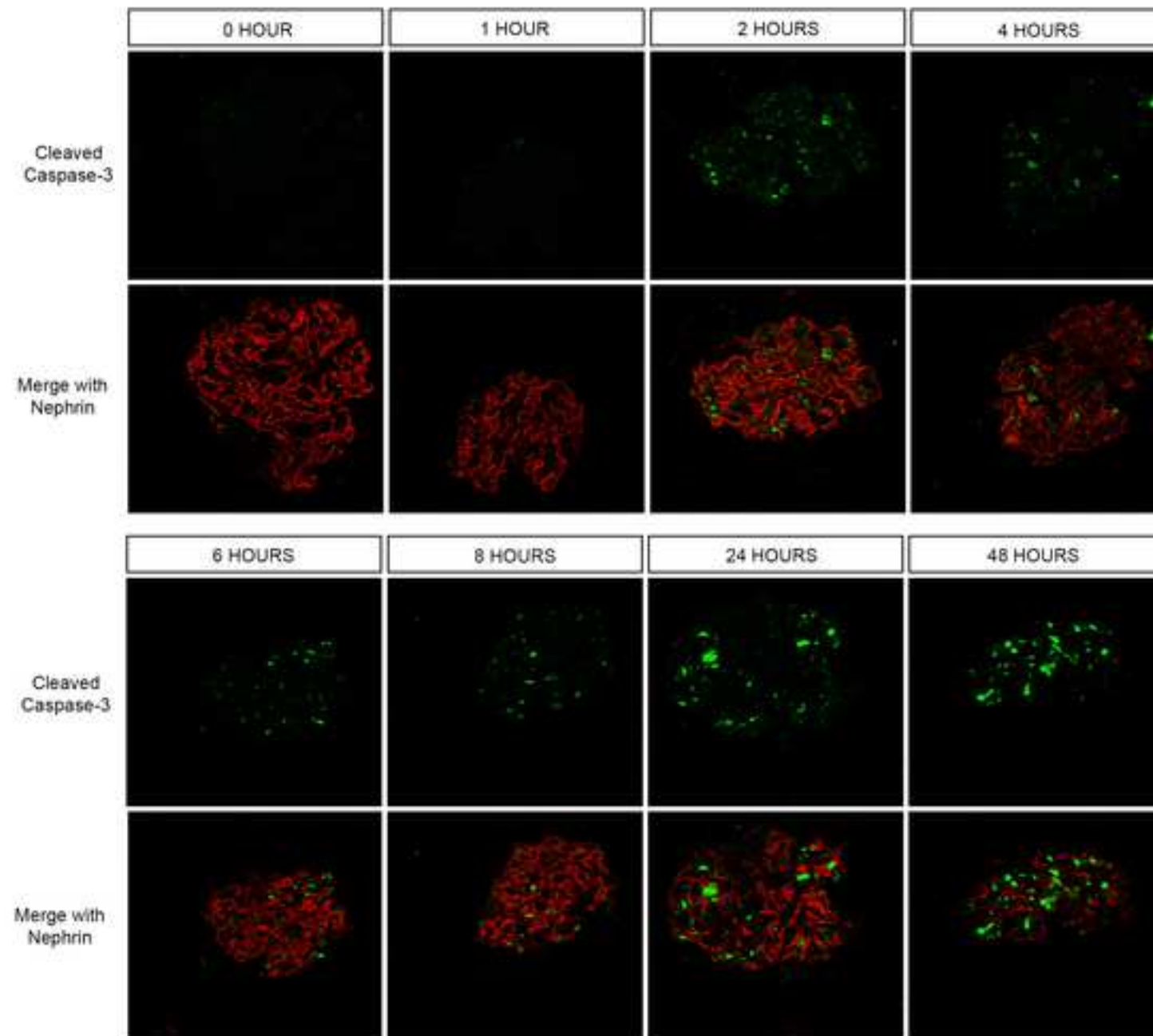




Figure 4



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
<b>Solutions, Chemicals, and Animals</b>			
Sprague-Dawley Rats	Envigo	207M	
Bovine serum albumin	Fisher	BP1605100	
Hank's Balanced Salt Solution with Ca2+ and Mg2+	Thermo	14025092	
1x Phosphate Buffered Saline	VWR	97063-660	
Protamine sulfate	MP Bio	ICN15197105	
Karnovsky's Fixative			
Gelatin			
Paraformaldehyde	EMD	EM-PX0055-3	
O.C.T.	Scigen	23730571	
RIPA Buffer			
Halt Protease Inhibitors	Thermo	PI78442	
Nephrin Antibody	Fitzgerald	2OR-NP002	
WT-1 Antibody	Abcam	ab89901-10ul	
PDGFR-β Antibody	Cell Signaling	#3169S	
CD31 Antibody	LSBio	LS-C348736	
Cleaved caspase-3 antibody	Cell Signaling	9661S	
Poly/Bed 812 (Luft) epoxy	Polysciences	08792-1	
<b>Equipment</b>			
Carbon dioxide chamber			
Conical tubes, 15 mL			
Conical tubes, 50 mL			
Surgical scissors			
Surgical forceps			
Sterile gauze			
Petri dishes, 100 mm			
Scalpels			
Razor blades			
Sterile filter apparatus			
Sieve Pan	Alfa Aesar	AA39999ON	
180um Sieve	Alfa Aesar	AA39996ON	
90um Sieve	Alfa Aesar	AA39990ON	
75um Sieve	Alfa Aesar	AA39991ON	
10 mL syringes			
600 mL beakers			
Cell culture incubator			
Picofuge			
Vortex			
Tissue Homogenizers	Fisher Scientific	50550226	
20 G needles			
Leica Reichart Ultracut			ultramicrotome



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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:*

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- 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.

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We have numbered the protocol steps according to JoVE instructions.

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- 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.*
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- 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.

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

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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 permeability 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 "Nepkin" and "um" in the TABLE OF MATERIALS/EQUIPMENT section. The authors should go through the manuscript carefully.*

We have corrected the typographic errors throughout.