

# Journal of Visualized Experiments

## Techniques to Assess Kidney Function in Mouse Models of Glomerular Disease --Manuscript Draft--

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|--|---|
| <b>Article Type:</b>   | Invited Methods Article - JoVE Produced Video   |
| <b>Section/Category:</b>   | JoVE Medicine   |
| <b>Manuscript Number:</b>  | JoVE57753   |
| <b>Full Title:</b>   | Techniques to Assess Kidney Function in Mouse Models of Glomerular Disease  |
| <b>Keywords:</b>   | glomerular phenotype; mouse models of renal disease; glomerular permeability; glomerular ultra-structure; mechanisms of glomerular function; VEGF-A |
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| <b>Additional Information:</b>   |   |
| <b>Question</b>  | <b>Response</b>   |
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15 December 2017

Dear Sir/Madam,

The incidence of chronic kidney disease is increasing around the world and places an important burden on health systems. There is therefore an acute need to increase research in kidney diseases and discover novel therapeutic targets.

To be able to do this, appropriate methods and techniques to assess the kidney function in animal models are needed. We present in this manuscript a minimal work-up for the kidney that we think is needed towards this goal. Sure, the list is not exhaustive and depending on the question asked there may be need for further experiments and techniques.

We hope you will find this paper of interest to your journal

Your sincerely,  
Dr Sebastian Oltean

# Techniques to Assess Kidney Function in Mouse Models of Glomerular Disease

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**Keywords:** glomerular phenotype, mouse models of renal disease, glomerular permeability, glomerular ultra-structure, mechanisms of glomerular function, VEGF-A

## SHORT ABSTRACT:

This protocol describes a full kidney work-up that should be carried out in mouse models of glomerular disease. The methods allow for detailed functional, structural, and mechanistic analysis of glomerular function, which can be applied to all mouse models of glomerular disease.

## **LONG ABSTRACT:**

The use of murine models to mimic human kidney disease is becoming increasingly common. This protocol describes a full kidney work-up that should be carried out in mouse models of glomerular disease, enabling a vast amount of information regarding kidney and glomerular function to be obtained from a single mouse. In comparison to alternative methods presented in the literature to assess glomerular function, the use of the method outlined in this paper enables the glomerular phenotype to be fully evaluated from multiple aspects. By using this method, the researcher can determine the kidney phenotype of the model and assess the mechanism as to why the phenotype develops. This vital information on the mechanism of disease is required when examining potential therapeutic avenues in these models. The methods allow for detailed functional assessment of the glomerular filtration barrier through measurement of the urinary albumin creatinine ratio and individual glomerular water permeability, as well as both structural and ultra-structural examination using the Periodic Acid Schiff stain and electron microscopy. Furthermore, analysis of the genes dysregulated at the mRNA and protein level enables mechanistic analysis of glomerular function. This protocol outlines the generic but adaptable methods that can be applied to all mouse models of glomerular disease.

## **INTRODUCTION:**

The use of murine models to mimic human kidney disease is becoming increasingly common. Such murine models include 1) spontaneous models such as spontaneously hypertensive rats (SHR)<sup>1</sup>, streptozotocin (STZ)-induced diabetic rats and mice<sup>2</sup>, and the db/db type II diabetic mice<sup>3</sup>, 2) genetically engineered models such as primary podocyte-specific focal segmental glomerular sclerosis (FSGS) models<sup>4</sup>, the podocyte-specific vascular endothelial growth factor A (VEGF-A) knock-out (VEGF-A KO) model<sup>5</sup>, and Alport syndrome models<sup>6</sup>, and 3) acquired models such as the 5/6 nephrectomy<sup>7</sup> and the unilateral ureteral obstruction (UUO) model<sup>8</sup>. In order to assess the different aspects of glomerular function in these models, several techniques are available. The purpose of this method paper is to demonstrate a comprehensive work-up that should be performed in mouse models of kidney disease in order to fully assess glomerular function.

The rationale behind the use of this method is that it enables the glomerular phenotype to be fully evaluated from multiple aspects. This includes assessing the glomerular permeability, both to protein and to water, the glomerular structural abnormalities, and changes in the expression/splicing of mRNAs and proteins essential for normal glomerular function. By using this method, the researcher is able to determine the kidney phenotype of the model and assess the mechanism as to why the phenotype develops. This is vital information on the mechanism of disease, which is required when examining potential therapeutic avenues in these models.

In the literature, it is a common occurrence to be presented with a mouse model of glomerular disease where the phenotype is determined by an increased level of albumin in the urine. However, there is evidence to suggest that a single method to determine glomerular function is not always effective; measuring the urinary albumin excretion rate or the urinary albumin creatinine ratio (uACR) only provides information on total renal function, and not of the individual glomeruli. Previous studies have demonstrated that the



permeability can vary in different glomeruli from the same kidney<sup>5,9,10</sup>. In addition, assessment of the permeability of individual glomeruli is a more sensitive way of assessing glomerular function; the technique of measuring the individual glomerular water permeability ( $L_pA/V_i$ ) has shown to be more sensitive to changes in glomerular function than measuring the uACR<sup>9</sup>. This assay would be beneficial in mouse models that are resistant to proteinuria, such as those on a c57BL/6 background<sup>11</sup>. The advantage of the present method paper is that it examines both the total renal permeability to albumin as well as the individual glomerular permeability to water.

Examination of glomerular structural abnormalities is often assessed by a battery of stains such as Periodic Acid Schiff (PAS), trichrome, and silver stains. These enable a trained renal pathologist to evaluate the level of renal disease via a scoring method. Although all good methods, changes to the glomerular macro-structure are not always observed in acute kidney injury models<sup>12</sup>. This method proposes that in addition to carrying out the renal histology techniques described above, the glomerular ultra-structure should also be assessed via electron microscopy (EM). A stained glomerulus can look relatively normal under a regular light microscope; however, upon assessment with EM, small changes in the glomerular basement membrane (GBM) width, podocyte foot process effacement, endothelial fenestrations, and the sub-podocyte space coverage can be analysed. Therefore, it is vital that both the glomerular ultra-structure and micro-structure is assessed to determine the mechanism of glomerular dysfunction.

In addition to assessing glomerular structural abnormalities, changes in mRNA and protein expression and splicing, as well as protein activation (e.g phosphorylation), should be examined to further elucidate the mechanisms of glomerular disease. When looking at glomerular disease, or, for example, when KO/over-expressing a gene specifically in glomerular cells, such as in the podocyte-specific VEGF-A KO mouse<sup>5</sup>, it is important that the protein and mRNA changes are examined only within the glomerular cells, and not the whole kidney. This protocol describes a method in which the glomeruli can be isolated from the mouse kidney cortex, and then the protein/RNA isolated. This allows specific analysis of the protein/mRNA dysregulation in the glomeruli of the disease model.

This protocol describes a full kidney work-up that should be carried out in mouse models of glomerular disease, enabling a vast amount of information regarding kidney and glomerular function to be obtained from a single mouse. The methods allow for detailed functional, structural, and mechanistic analysis of glomerular function, which can be applied to all mouse models of glomerular disease.

## PROTOCOL

All experiments were conducted in accordance with UK legislation and local ethical committee approval. Animal studies were approved by University of Bristol research ethics committee.

### Assessment of glomerular phenotype in mouse models of glomerular injury

#### 1. Urinary albumin creatinine ratio (uACR)

1. 1) Urine should be collected at baseline and at regular intervals.

1. 2) Set up mouse metabolic cages with water and enrichment diet. Place mice in individual cages for 6 hr in a quiet room.

1. 3) Return mice to regular housing and collect urine from the empty cages. A minimum of 50  $\mu$ l is required.

1. 4) Centrifuge urine at 500 x g for 10 min and discard sediment. Urine can be stored at -20 °C in the short-term at this point.

1. 5) Dilute the urine into 1% BSA in 1x PBS at a 1:500 to 1:10000 dilution, depending on the severity of the albuminuria; the end volume should be >400  $\mu$ l. This will take some optimization to determine the right dilution at each time point.

1. 6) Quantify the urinary albumin concentration using the mouse albumin ELISA kit (Bethyl Laboratories, Inc.) per the kit instructions. Run samples in triplicate. Determine the albumin concentration of each sample by reading the plate at an absorbance of 450 nm. The standard curve is generated from the standards and is used to quantify the albumin present in each sample

1. 7) Using the original urine sample, dilute the urine 1:1 to 1:10 with dH<sub>2</sub>O; the end volume should be >70  $\mu$ l. This will take some optimization to determine the right dilution of each sample.

1. 8) Quantify the urinary creatinine concentration using the creatinine companion kit (Exocell) per the kit instructions. Run samples in triplicate. Determine the creatinine concentration of each sample by reading the plate at an absorbance of 490 nm before and after the addition of the acid solution (CAUTION, corrosive; avoid contact with skin) . The difference between these absorbance values is directly proportional to the creatinine concentration in each sample. A standard curve is generation from the standards.

1. 9) Generate the uACR ( $\mu$ g/mg). Data can be normalized to the baseline value of each mouse for graphical representation.

## 2. Tissue and Blood Collection

2. 1) Prepare the following solutions: fresh 2.5% glutaraldehyde (CAUTION, toxic, sensitizer, irritant; use in a fume cabinet) in 0.1 M sodium cacodylate (CAUTION, toxic, use in a fume cabinet) (pH 7.3), 4% PFA (CAUTION, fixative; use in fume cabinet) in 1x PBS, mammalian Ringer (115 mM NaCl, 10 mM sodium acetate, 1.2 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 MM NaHCO<sub>3</sub>, 1.2 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 5.5 mM D(+)glucose, pH 7.4) with 1% BSA, and 1x PBS.

2. 2) The following materials are required: isoflurane, small anti-coagulant-coated blood tubes, 23-25G needles, 5 ml anti-coagulant coated syringes, 10 ml glass vials, 10 ml plastic vials, 0.5 ml plastic tubes, disposable tissue molds, dry ice, liquid N<sub>2</sub>, mouse surgical tools, and optimal cutting medium (OCT).

2. 3) Place the mouse under deep anesthesia using an isoflurane chamber. Cull mouse via cardiac puncture into the left ventricle and collect as much blood as possible. Transfer to the anti-coagulant coated blood tube for up to 4 hr.

2. 5) Dissect out the kidneys and wash in 1x PBS.

2. 6) EM; remove one pole of kidney cortex and cut into 1 mm<sup>3</sup> pieces. Place in 5 ml 2.5% glutaraldehyde solution in a glass EM vial. Store at 4 °C and process within 1 month for best results.

2. 7) Histology; remove one pole of kidney cortex and fix in 5 ml 4% PFA at 4 °C for 24 hrs. Transfer to 5 ml 70% EtOH for 24 hours before embedding in paraffin.

2. 8) Immunofluorescence; place a 3 mm<sup>3</sup> piece of kidney cortex into the tissue mold and coat in OCT. Place on dry ice to freeze and store at -80 °C.

2. 9) Protein and RNA; place 3x 2 mm<sup>3</sup> pieces of kidney cortex into 0.5 ml plastic tubes and snap freeze in liquid N<sub>2</sub>. Store at -80 °C.

2. 10) Isolation of glomeruli; slice up the remaining kidney tissue and place in 5 ml mammalian Ringer with 1% BSA on ice. Prepare to sieve glomeruli immediately.

### 3. Plasma creatinine

3. 1) Centrifuge the blood sample at 500 x g for 15 min at 4 °C.

3. 2) Collect the plasma, which can be stored at -20 °C in the short-term at this point.

3. 3) Quantify the plasma creatinine concentration using the creatinine companion kit (Exocell) per the kit instructions. Run samples in triplicate. Determine the creatinine concentration of each sample by reading the plate at an absorbance of 490 nm before and after the addition of the acid solution. The difference between these absorbance values is directly proportional to the creatinine concentration in each sample. A standard curve is generated from the standards.

### 4. Isolation of glomeruli

4. 1) Using the kidney tissue placed in mammalian Ringer with 1% BSA, dissect the glomeruli using a standard sieving technique<sup>13</sup>.

4. 2) The glomerular harvest retained by the 100 µm and 70 µm sieves is transferred to 10 ml fresh mammalian Ringer solution with 1% BSA, on ice.

4. 3) Remove 5 ml of the solution containing glomeruli to two separate tubes (2.5 ml each) and centrifuge at 1000 x g for 10 min at 4 °C. Remove the supernatant and snap freeze the glomeruli in liquid N<sub>2</sub> before storing at -80 °C for later protein and RNA extraction.

4. 4) The remaining solution containing glomeruli is placed in a water bath at 37 °C for measurement of the glomerular  $L_pA/V_i$  *ex vivo*. This must be completed within 3 hr of removing the kidney.

#### 5. Glomerular water permeability ( $L_pA/V_i$ )

5. 1) Solutions required: mammalian Ringer with 1% BSA (pH 7.4), mammalian Ringer with 8% BSA (pH 7.4). Both should be warmed to 37 °C.

5. 2) Micropipettes are pulled from glass capillary tubes (o.d. 1.2 mm; Harvard Apparatus), and a 5-8  $\mu$ m aperture tip generated.

5. 3) The glomerular  $L_pA/V_i$  rig is set up as described in Salmon et al<sup>10</sup>.

5. 4) Intact individual glomeruli that are free of Bowman's capsule and tubular fragments are caught onto the micropipette using suction. A detailed summary of the oncometric assay can be found in Salmon et al. (2005). In brief, whilst recording the glomerulus secured on the micropipette, the glomerulus is equilibrated in the 1% BSA Ringer solution for 30 sec before switching the perfusate to the concentrated 8% BSA Ringer solution for 10 sec. The perfusate is then switched back to 1% BSA Ringer and the recording stopped. The glomerulus is washed away and the process is repeated.

5. 5) With the change in the oncometric gradient when switching to the 8% BSA Ringer solution, the size of the glomerulus visually shrinks as the water moves out of the capillaries. This shrinkage is used to calculate the glomerular water permeability ( $L_pA$ ) normalized to the glomerular volume ( $V_i$ ). Detailed information regarding analysis can be found in Salmon et al<sup>10</sup>.

#### 6. Periodic Acid Schiff (PAS) stain

6. 1) Section PFA-fixed kidney cortex, embedded in paraffin, using a microtome at 5  $\mu$ m thickness onto slides. Dry at 37 °C for 1 hr.

6. 2) Deparaffinize slides by incubating twice in xylene (CAUTION, irritant; use in fume cabinet) for 3 min each, twice in 100% EtOH 3 min each, and then once in 95%, 70%, and 50% EtOH for 3 min each. Re-hydrate slides in dH<sub>2</sub>O.

6. 3) Incubate slides in periodic acid solution (CAUTION, irritant; use in fume cabinet) (1 g/dl) for 5 min, then rinse slides in several changes of dH<sub>2</sub>O.

6. 4) Incubate slides in Schiff's reagent (Parasoaniline HCl 6 g/l and sodium metabisulfite 4% in HCl 0.25 mol/l) for 15 min. Wash slides in running tap water for 5 min.

6. 5) Counterstain with Hematoxylin for 3 sec before thoroughly rinsing slides in running tap water.

6. 6) Dehydrate slides using the reverse of the deparaffinization protocol in 6. 2), ending with xylene.

6. 7) Air dry slides and mount with xylene-based mounting media.

6. 8) Image on a light microscope at 400 x magnification to assess glomerular structures.

## 7. Transmission electron microscopy (EM)

7. 1) Take the 2.5% gluteraldehyde- fixed diced kidney and post-fix in 1% osmium tetroxide for 1 hr. Wash in 0.1 M cacodylate buffer (pH 7.3) and then dH<sub>2</sub>O (3 x 15 min changes).

7. 2) Dehydrate with EtOH and embed in Araldite resin (Agar Scientific).

7. 3) Cut sections at 50-100 nm thickness and stain with 3% (aqueous) uranyl acetate and Reynolds' lead citrate solution.

7. 4) Take digital micrographs over several areas of the glomerulus to be sure the podocytes, GEnCs, GBM, and mesangium can be identified.

7. 5) Use Image J to analyze the blinded glomeruli. Set the scale for each micrograph. Use the protocols listed below for measurement of each parameter:

7. 4. 1) GBM: Insert a grid (10 x 10) over the micrograph and measure the thickness of the GBM at the point where the grid lines cross the GBM.

7. 4. 2) Endothelial fenestration number: count the number of fenestrations per unit length of GBM.

7. 4. 3) Podocyte foot process width: Insert a grid (10 x 10) over the micrograph and measure the width of the podocyte foot processes that cross the grid lines.

7. 4. 4) Podocyte slit width: Insert a grid (10 x 10) over the micrograph and measure the width of the podocyte slit diaphragms that cross the grid lines.

7. 4. 5) Number of podocyte foot processes: count the number of foot processes per unit length of GBM.

7. 4. 6) sub-podocyte space coverage: see detailed method in Neal et al<sup>14</sup>.

## 8. Immunofluorescence for podocyte and endothelial markers

8. 1) Place the OCT-mold containing frozen kidney cortex at -20 °C 2 hr prior to sectioning.

8. 2) Using a cryostat, section tissue at a 5 µm thickness onto poly-l-lysine coated slides.

8. 3) Upon removal from the cryostat, fix slides in 4% PFA for 10 min. Wash slides 3 x 5 min in dH<sub>2</sub>O.

8. 4) To minimize the amount of antibody used, draw around sections with a hydrophobic pen. Do not let sections dry.

8. 5) Incubate in blocking solution (3% BSA and 5% normal serum in 1x PBS) for 1 hr at room temperature.

8. 6) Remove the blocking solution with an aspirator and incubate sections with primary antibody (Nephrin [Acris], podocin [Sigma], or PECAM-1 [BD Biosciences]) diluted 1:250 (3% BSA in 1x PBS). Place slides in a humidified chamber at 4 °C overnight.

8. 7) Wash slides 3 x 5 min in 1x PBS.

8. 8) Incubate with appropriate fluorescent secondary antibody dilution 1:1000 (3% BSA in 1x PBS) for 2 hr at room temperature.

8. 9) Wash slides 3 x 5 min in 1x PBS. Mount with fluorescent mounting media containing DAPI.

8. 10) Image slides with a fluorescent microscope at 400 x magnification to view the glomeruli.

8. 11) Analyze the staining intensity, normalized to the glomerular area, and pattern, i.e. number of capillary loops normalized to the glomerular area, using Image J.

## **9. Protein extraction and Western blotting**

9. 1) Protein can be extracted from kidney cortex and sieved glomeruli; the protocol is the same for each and the volume of lysis buffer should be adjusted for the amount of tissue.

9. 2) Thaw kidney/glomeruli on ice before adding NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris pH 8) containing protease and phosphatase inhibitors. Homogenize the sample for 30 sec.

9. 3) Incubate homogenized samples on ice for 30 min; vortex at regular intervals.

9. 4) Centrifuge samples at 10,000 x g for 15 min at 4 °C.

9. 5) Remove supernatant to a fresh tube on ice.

9. 6) Denature proteins using the standard 2x Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl, pH 6.8) at a 1:1 ratio. Boil the mixture at 95-100 °C for 5 min.

9.7) Assess the expression of glomerular cell marker proteins (Nephrin, Podocin, PECAM-1, etc) and the phosphorylation and expression of proteins known/hypothesized to be altered in the kidney/glomeruli of the disease model using Western blotting (standard method; Mahmood and Yang<sup>15</sup>). The protocol will vary depending on the size and abundance of the protein of interest.

## **10. RNA extraction and polymerase chain reaction (PCR)**

10. 1) Whilst the kidney cortex is still frozen, thoroughly grind in 3 ml of TRIzol reagent (CAUTION, irritant; use in fume cabinet) (ThermoFisher) using a pestle and mortar. If using glomerular extracts, add 1 ml of TRIzol reagent (ThermoFisher) and homogenize the sample for 30 sec.
10. 2) Perform an RNA extraction using the method described by Chomczynski and Sacchi<sup>16</sup>.
10. 3) Assess the quantity and quality of RNA obtained using one of the various methods available. RNA can be aliquoted and stored at -80 °C at this point. Avoid repeat freeze thawing.
10. 4) DNase treat 1 µg RNA (make volume up to 10 µl with RNase-free water plus 1 µl DNase and 1 µl DNase buffer) for 1 hr at 37 °C. Stop the reaction with 1 µl DNase stop solution at 65 °C for 10 min.
10. 5) Add 0.5 µl oligo (dT) and random primers. Incubate at 70 °C for 10 min.
10. 6) Immediately quench on ice for 5 min.
10. 7) Add the following; MMLV reverse transcriptase enzyme (400 U; replace with DEPC H<sub>2</sub>O in the RT - control sample), MMLV buffer (1x), dNTP mix (0.5 mM), and ribonuclease inhibitor (40 U); make up to 50 µl with DEPC water.
10. 8) Incubate reaction mix at 37 °C for 1 hr followed by 95 °C for 5 min to deactivate the enzyme.
10. 9) Assess the quantity and quality of cDNA using the various methods available.
10. 10) cDNA can now be used in PCR to assess the mRNA expression and splicing patterns of genes hypothesized to be dysregulated in the glomerular disease model. The protocol will vary depending on the gene of interest.

## REPRESENTATIVE RESULTS

### **Progressive depletion of podocyte VEGF-A results in albuminuria, which is rescued by the constitutive expression of the human VEGF-A<sub>165b</sub> splice isoform**

Urine was collected using metabolic cages from wild type (WT), inducible podocyte-specific VEGF-A knock out (VEGF-A KO), and VEGF-A KO X Neph-VEGF<sub>165b</sub> mice (VEGF-A KO mice that over-express the human VEGF-A<sub>165b</sub> isoform in the podocytes in a constitutive manner). Upon measurement of the urinary albumin creatinine ratio at weeks 0, 4, 10, and 14 after doxycycline induction of VEGF-A KO, VEGF-A KO mice developed progressive albuminuria by 10 weeks compared to WT littermate controls. The absolute values can be seen in Figure 1A, and the normalized to the baseline of each mouse values in Figure 1B. However, albuminuria is not observed in the VEGF-A X Neph-VEGF-A<sub>165b</sub> mice (Figure 1), indicating that VEGF-A<sub>165b</sub> is protective in the model of albuminuria<sup>5</sup>.

**Progressive depletion of podocyte VEGF-A results in increased glomerular  $L_pA/V_i$ , which is rescued by the constitutive expression of the human VEGF-A<sub>165b</sub> splice isoform**

The glomerular  $L_pA/V_i$  was measured in individual glomeruli sieved from WT, VEGF-A KO, and VEGF-A X Neph-VEGF-A<sub>165b</sub> kidneys. An example of how a glomerulus is caught and the shrinkage observed when perfused with 8% BSA is shown in Figure 2A. This shrinkage is then used to determine the glomerular  $L_pA/V_i$  for each glomerulus (Figure 2B). VEGF-A KO mice had a significantly increased glomerular  $L_pA/V_i$  at 14 weeks post VEGF-KO induction compared to WT control glomeruli. Although lower in VEGF-A X Neph-VEGF-A<sub>165b</sub> mice, the increased glomerular  $L_pA/V_i$  was not prevented by over-expression of VEGF-A<sub>165b</sub> at 14 weeks<sup>5</sup>.

**VEGF-A KO mice develop ultra-structural abnormalities, some of which are prevented by over-expression of VEGF-A<sub>165b</sub>**

PAS staining of kidney cortex sections 14 weeks after induction of VEGF-A KO did not reveal any glomerular structural abnormalities in the VEGF-A KO or VEGF-A X Neph-VEGF-A<sub>165b</sub> mice (Figure 1A). However, upon analysis of the glomerular ultra-structure via EM, VEGF-A KO mice had developed an increased GBM width, decreased number of endothelial fenestrae, decreased SPS coverage, and increased average podocyte slit width (Figure 3C - F). The average podocyte foot process width and number of slits remained unchanged (Figure 3B and G). Over-expression of VEGF-A<sub>165b</sub> in the VEGF-A KO mice prevented the changes to the GBM and slit width (Figure 3C and F). However, VEGF-A<sub>165b</sub> had no effect on the altered fenestrae number and SPS coverage (Figure 3D and E)<sup>5</sup>.

**The mRNA and protein expression levels of genes can be assessed from sieved glomeruli**

RT-PCR performed on RNA extracted from sieved glomeruli revealed that the human VEGF-A<sub>165b</sub> mRNA is only present in the VEGF-A KO X Neph-VEGF-A<sub>165b</sub> mice (Figure 4A). When extracting protein from sieved glomeruli and assessing the levels of proteins via Western blotting, the glomerular protein expression of VEGFR-2 was found to be decreased in VEGF-A KO mice, which was prevented by over-expression of VEGF-A<sub>165b</sub> (Figure 4B and C)<sup>5</sup>.

**Legends to Figures**

**Figure 1. (A)** The uACR values at weeks 0, 4, 10, and 14 after induction of VEGF-A KO in WT, VEGF-A KO, and VEGF-A X Neph-VEGF-A<sub>165b</sub> mice. **(B)** The same uACR values normalized to the baseline value (week 0) of each individual mouse. The uACR significantly increased in VEGF-A KO mice at weeks 10 and 14 compared to WT littermate controls, which was prevented in the VEGF-A X Neph-VEGF-A<sub>165b</sub> mice (\* $p < 0.05$ ; Two way ANOVA, correction for comparison between pairs;  $n = 4-12$  mice per time point).

**Figure 2. (A)** A glomerulus is caught on the micropipette via suction; the perfusate is switch from 1% BSA (Ai) to 8% BSA (Aii), and glomerular shrinkage is observed. **(B)** Measurements taken before and after the 8% BSA switch are used to determine the glomerular  $L_pA/V_i$ . **(C)** VEGF-A KO mice develop an increased glomerular  $L_pA/V_i$  at 14 weeks post induction of



VEGF-A KO compared to WT controls. This was not significantly prevented in VEGF-A X Neph-VEGF-A<sub>165b</sub> mice (\*p<0.05; One way ANOVA, Bonferroni correction for comparison between pairs; n=4-9 mice, 15-30 glomeruli).

**Figure 3. (A)** PAS staining of the kidney cortex did not indicate any structural abnormalities in the glomeruli from WT, VEGF-A KO, and VEGF-A X Neph-VEGF-A<sub>165b</sub> mice (Ai – iii). EM revealed ultra-structural abnormalities in the VEGF-A KO glomeruli (Aiv – vi). **(B)** The average FPW did not change between groups. **(C)** The GBM increased in the VEGF-A KO glomeruli, which was prevented by VEGF-A<sub>165b</sub>. **(D)** The number of fenestrae was decreased in VEGF-A KO glomeruli, which remained unaltered by VEGF-A<sub>165b</sub>. **(E)** The SPS coverage was decreased in VEGF-A KO glomeruli, which also remained unchanged by VEGF-A<sub>165b</sub>. **(F)** The average slit width increase in VEGF-A KO glomeruli, which was prevented by VEGF-A<sub>165b</sub>. **(G)** The slit number was unchanged between the three groups (\*p<0.05; One way ANOVA, Bonferroni correction for comparison between pairs; n=3 mice, 9 glomeruli).

**Figure 4. (A)** RT-PCR showed that human VEGF-A<sub>165b</sub> mRNA expression was only evident in the sieved glomeruli from VEGF-A KO X Neph-VEGF-A<sub>165b</sub> mice. **(B)** Western blotting indicated that VEGFR-2 protein expression was down-regulated in VEGF-A KO glomeruli, which was prevented in VEGF-A KO X Neph-VEGF-A<sub>165b</sub> glomeruli (\*p<0.05; One way ANOVA, Bonferroni correction for comparison between pairs; n=3-6 mice).

## DISCUSSION

This protocol describes a full kidney work-up that should be carried out in mouse models of glomerular disease, enabling a vast amount of information regarding kidney and glomerular function to be obtained from a single mouse. The critical steps in each method allow for detailed functional, structural, and mechanistic analysis of glomerular function, including assessment of the permeability of the kidneys as a whole (uACR and plasma creatinine measurements), the permeability of individual glomeruli (glomerular L<sub>p</sub>A/V<sub>i</sub>), examination of the structural alterations (PAS, Trichrome blue, and EM), protein localization (IF), and glomerular gene expression (RT-PCR and Western blotting). These methods are key to the full assessment of glomerular function in mouse models of renal disease.

When assessing the permeability of the GFB, many studies have opted to use the conventional uACR or 24 hr albumin excretion rate as an effective measure<sup>17,18</sup>. Although these techniques allow assessment of the GFB permeability as a whole, it does not allow for individual glomerular permeability assessment and variation amongst glomeruli. Previous studies have found measurement of the glomerular L<sub>p</sub>A/V<sub>i</sub> to be a more sensitive measure of changes to the GFB permeability<sup>5,9</sup>. Indeed, in the representative results demonstrated in this paper, at 14 weeks post induction of VEGF-A KO, VEGF-A KO X Neph-VEGF-A<sub>165b</sub> mice have a significantly lower uACR compared to VEGF-A KO mice; however, this results is not reflected in the glomerular L<sub>p</sub>A/V<sub>i</sub> measurements, where VEGF-A<sub>165b</sub> did not significantly prevent increases in the GFB permeability (Figure 1 and 2)<sup>5</sup>. This shows the importance of using multiple assays to assess both the kidney permeability and the permeability of individual glomeruli. Furthermore, the glomerular L<sub>p</sub>A/V<sub>i</sub> oncometric assay suggests that the permeability of individual glomeruli from the same kidney can vary greatly, especially in disease models<sup>5,10,19</sup>. One limitation to measuring the glomerular L<sub>p</sub>A/V<sub>i</sub> is that it can only be

performed at the experimental end-point; thus, regular uACR measurements are required to give an indication of the experimental end-point.

In addition to assessing the functional phenotype, the present method also encourages assessment of the structural and ultrastructural phenotype. This can be done using a selection of stains such as PAS, trichrome, and silver stains; each to assess different aspects of the glomerular morphology. In acute models of glomerular disease, which is often the case in mouse models, it can be difficult to detect any major structural abnormalities using these stains unless you are a trained renal pathologist. Therefore, carrying out EM is suggested to assess the ultrastructure of the GFB, which allows the quantitative measurement of parameters such as the GBM, endothelial fenestrae size and number, and podocyte characteristics. Such measurements require minimal training to perform and enables the investigator to determine the cell-types/structures affected in a disease model. In the example shown in the representative results, the VEGF-A KO mouse was found to be a mild model of glomerular disease, thus, no major structural abnormalities were present upon PAS staining. However, podocyte-specific VEGF-A KO did induce changes to the GBM, podocytes, and endothelial cells when examining the glomerular ultra-structure<sup>5</sup>. Unfortunately, the preparation of the kidney for EM described in the present method does not enable detection of the endothelial glycocalyx, which is also known to have significant effects on the permeability of the GFB<sup>19</sup>. In order to accurately measure the glycocalyx depth, the kidney should be perfuse-fixed with 2.5% glutaraldehyde with 1% Alcian blue for endothelial glycocalyx labelling, as described in Oltean et al<sup>19</sup>.

Once the functional and structural phenotype have been assessed, the expression/activation patterns of different genes and pathways can then be assessed specifically in the glomeruli. Prior ultra-structural assessment could give some information regarding the cell types/glomerular structures involved, indicating whether podocyte- or endothelial-specific genes/pathways should be examined. For example, in the representative results from the VEGF-A KO mice, a reduction in the endothelial fenestrae number was observed (Figure 3D); therefore, the glomerular protein expression of an endothelial marker known to be involved in the VEGF-A pathway was examined; VEGFR-2 (Figure 4B)<sup>5</sup>. In addition to the expression of proteins in the glomeruli, their localization can also be visualized using IF. In a study by Zhang et al.<sup>20</sup>, podocyte-specific overexpression of GLUT1 was confirmed in the podocytes by IF co-localizing the increased GLUT1 with podocin.

In comparison to alternative methods presented in the literature to assess glomerular function, the use of the method outlined in this paper to assess kidney function in mouse models of glomerular disease enables the glomerular phenotype to be fully evaluated from multiple aspects. By using this method, the researcher is able to determine the kidney phenotype of the model and assess the mechanism as to why the phenotype develops. This vital information on the mechanism of disease is required when examining potential therapeutic avenues in these models. This method can be easily applied to future investigations into glomerular function both in the assessment of disease phenotypes and potential therapeutics.

In conclusion, this generic and adaptable protocol describes a full kidney work-up for mouse models of glomerular disease, enabling a vast amount of information regarding kidney and glomerular function to be obtained from a single mouse. The methods allow for detailed functional, structural, and mechanistic analysis of glomerular function, which can be applied to all mouse models of glomerular disease.

## ACKNOWLEDGEMENTS

This work was supported by the British Heart Foundation, Richard Bright VEGF Research Trust and the MRC.

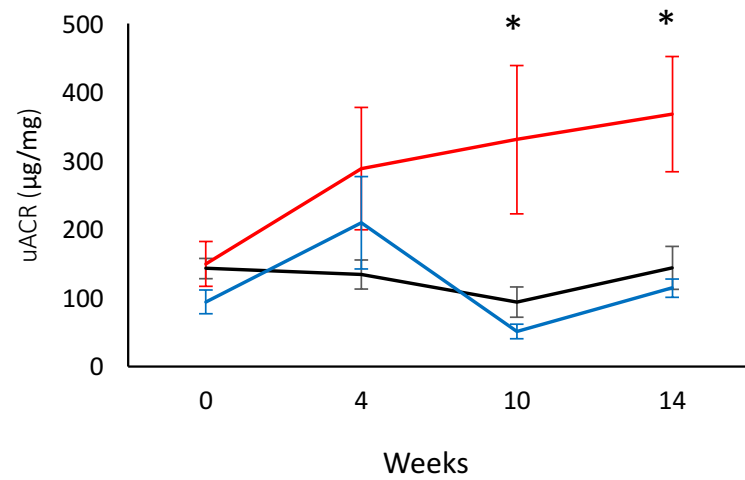
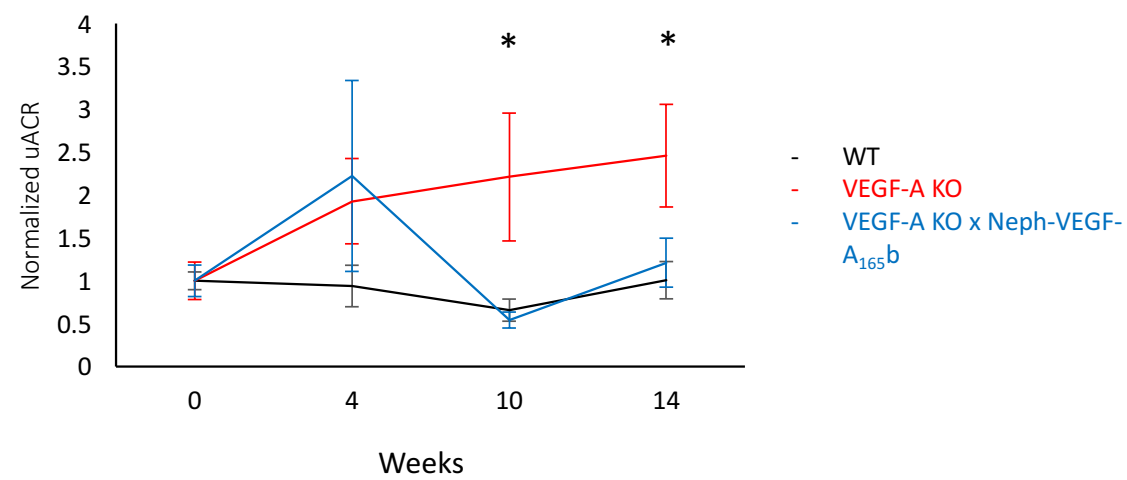
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The authors have nothing to disclose.

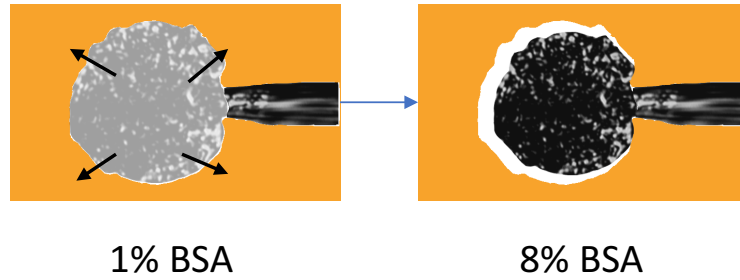
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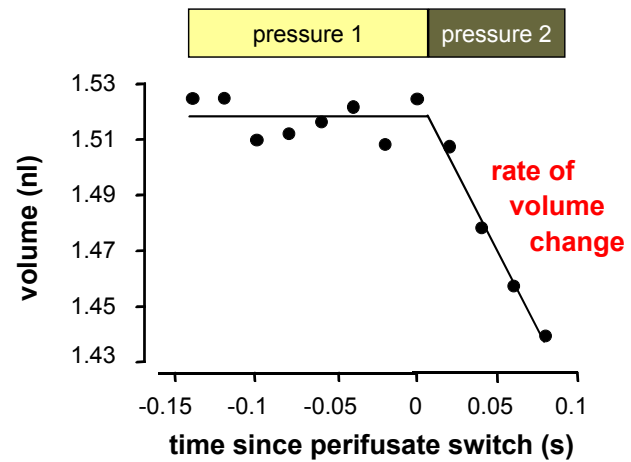
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**A****B**

**A**



**B**



**rate of volume change**

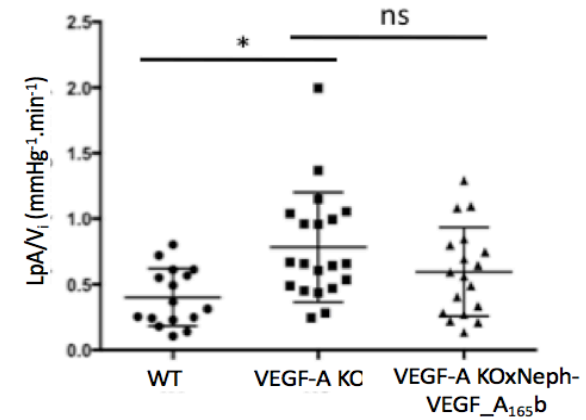
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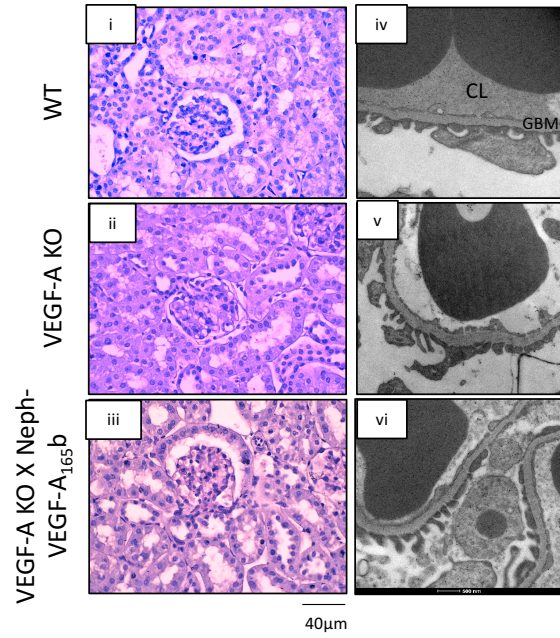
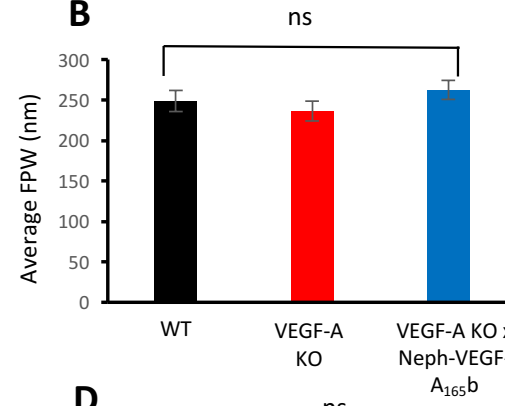
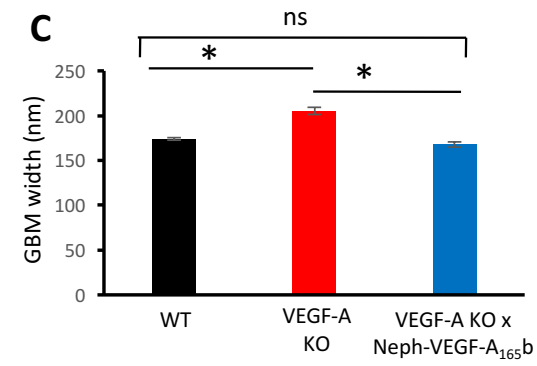
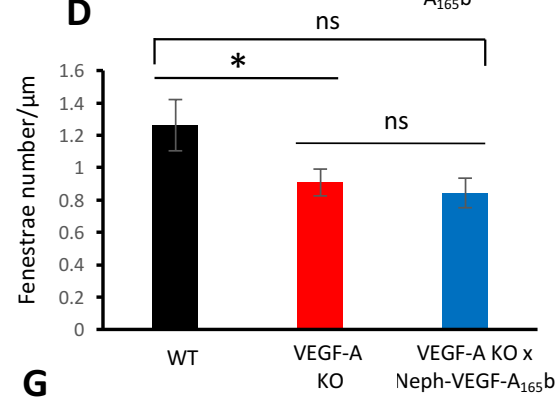
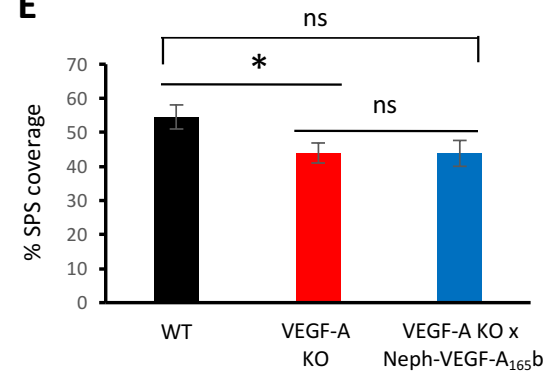
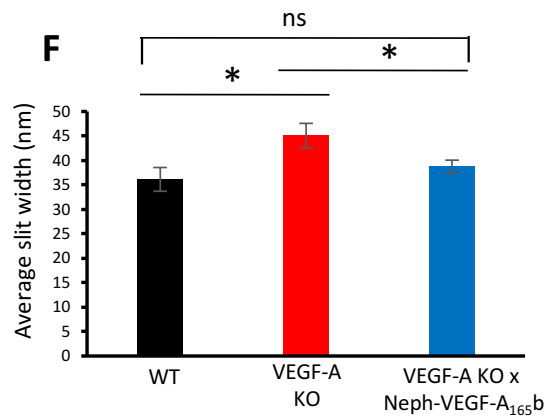
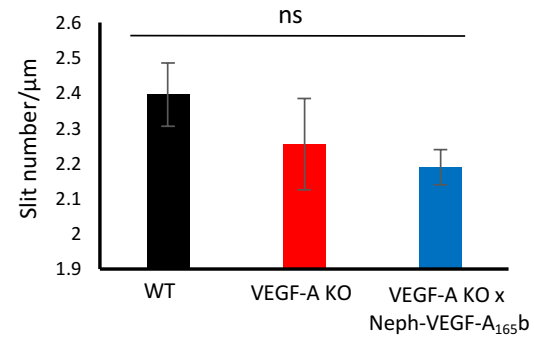
= water permeability

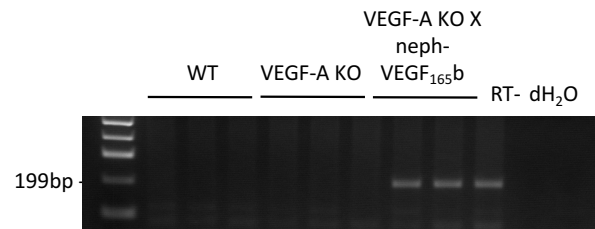
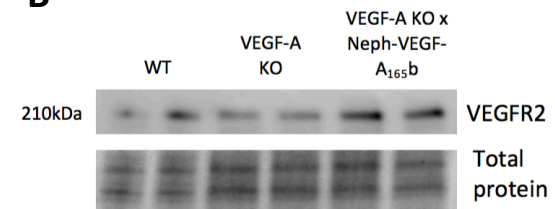
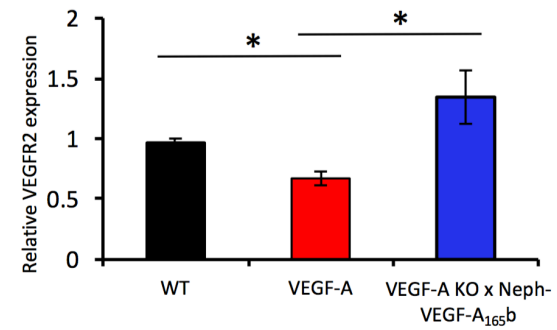
= ultrafiltration coefficient

=  $L_pA$

**C**



**A****B****C****D****E****F****G**

**A****B****C**



| Name of Material/ Equipment                | Company             | Catalog Number   | Comments/Description  |
|--|---------------------|------------------|---|
| Metabolic Cages                            | Harvard Apparatus   | 52-6731          | Built at the Univeristy of Bristol - not commercially available |
| Mouse Albumin ELISA Quantitation Set       | Bethyl Laboratories | E90-134          |   |
| Creatinine Companion                       | Exocell             | 1012 Strip Plate |   |
| Glass Capillary Tubes                      | Harvard Apparatus   | EC1 64-0770      |   |
| Glomerular Permeability Rig                |                     |                  |   |
| 100 µm Stainless Steel Sieve               | Cole-Parmer         | WZ-59984-18      |   |
| 70 µm Stainless Steel Sieve                | Cole-Parmer         | WZ-59984-21      |   |
| Periodic Acid-Schiff (PAS) Staining System | Sigma-Aldrich       | 395B-1KT         |   |
| Hematoxylin                                | Sigma-Aldrich       | H3136            |   |
| Poly-Prep Slides                           | Sigma-Aldrich       | P0425-72EA       |   |
| Nephrin (1243-1256) Antibody               | Acris               | BP5030           |   |
| Anti-Podocin                               | Sigma-Aldrich       | P0372-200UL      |   |

|  |                         |          |
|--|-------------------------|----------|
| Anti-CD31  | BD Biosciences          | 550274   |
| NP40 Cell Lysis Buffer                           | ThermoFisher Scientific | FNN0021  |
| Halt Protease and Phosphatase Inhibitor Cocktail | ThermoFisher Scientific | 78437X4  |
| TRIzol   | ThermoFisher Scientific | 15596018 |
| Dnase I  | New England Biolabs     | M0303S   |
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