Video Article

Isolation of Glomeruli and *In Vivo* Labeling of Glomerular Cell Surface Proteins

Eva Königshausen[1], Sebastian A. Potthoff[1], Raphael Haase[1], Catherine Meyer-Schwesinger[2], Ernest Kaufmann[1], L. Christian Rump[1], Johannes Stegbauer[1], Lorenz Sellin[1], Ivo Quack[1], Magdalena Woznowksi[1]

[1]Department of Nephrology, Medical Faculty, Heinrich-Heine-University

[2]III. Medical Clinic, University Hospital Eppendorf

Correspondence to: Eva Königshausen at eva.koenigshausen@med.uni-duesseldorf.de

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Abstract

Proteinuria results from the disruption of the glomerular filter that is composed of the fenestrated endothelium, glomerular basement membrane, and podocytes with their slit diaphragms. The delicate structure of the glomerular filter, especially the slit diaphragm, relies on the interplay of diverse cell surface proteins. Studying these cell surface proteins has so far been limited to *in vitro* studies or histologic analysis. Here, we present a murine *in vivo* biotinylation labeling method, which enables the study of glomerular cell surface proteins under physiologic and pathophysiologic conditions. This protocol contains information on how to perfuse mouse kidneys, isolate glomeruli, and perform endogenous immunoprecipitation of a protein of interest. Semi-quantitation of glomerular cell surface abundance is readily available with this novel method, and all proteins accessible to biotin perfusion and immunoprecipitation can be studied. In addition, isolation of glomeruli with or without biotinylation enables further analysis of glomerular RNA and protein as well as primary glomerular cell culture (*i.e.,* primary podocyte cell culture).

Introduction

Proteinuria is a hallmark of glomerular injury and usually accompanies disruption of the glomerular filter[1]. The glomerular filter is composed of the fenestrated endothelium, glomerular basement membrane, and podocytes. The delicate molecular structure of the glomerular filter is highly dynamic and subject to cell surface protein trafficking in both healthy and diseased kidneys[2,3,4,5,6]. Endocytosis of cell surface proteins has been shown to be essential for the survival of podocytes[7]. Nephrin and podocalyxin are transmembrane proteins expressed on podocytes. Nephrin is the backbone of the glomerular slit diaphragm, while podocalyxin is a sialoglycoprotein coating the secondary foot processes of podocytes[8,9,10]. Endocytic trafficking has previously been shown for nephrin and podocalyxin[3,11,12,13,14].

To the best of our knowledge, endocytosis of cell surface proteins has not yet been described in glomerular endothelial cells in the literature. However, endothelial cells in general express all necessary proteins for the different types of endocytosis (*i.e.,* clathrin-dependent, raft-dependent endocytosis)[15,16]. Therefore, endothelial cell surface trafficking may be studied with this method using, for example, vascular endothelial (VE)-cadherin and intracellular adhesion molecule (ICAM-2) as a cell surface marker protein for glomerular endothelial cells[17].

Unfortunately, there is no accurate *in vitro* model for the delicate three-layered glomerular filter in which cell surface protein trafficking can be studied. The goal of this method is thus to study glomerular protein trafficking *in vivo*. In addition, this protocol contains information on how to isolate glomeruli, enabling further analysis of glomerular RNA, proteins, or cells. Similar glomerular isolation techniques have been described by different groups[18,19].

Previously, we and others have used *ex vivo* labeling of glomerular cell surface proteins by biotinylation[2,3,4,20,21]. However, in this *ex vivo* method, isolated glomeruli were exposed to mechanical stress, which may influence endocytic trafficking. Alternatively, immunofluorescence labeling of glomerular cell surface proteins has extensively been used in the literature[2,20,22]. With this method, however, only a small number of proteins can be analyzed within one slide, and quantitation of immunofluorescence images is often difficult.

This novel *in vivo* method offers a reliable tool to study glomerular cell surface protein abundance and trafficking accurately in healthy and diseased kidneys, and it can be used as an addition to immunofluorescence tests.

Protocol

Mice were obtained as an in-house breed from the local animal care facility or from Janvier Labs in France. The investigations were conducted according to the guidelines outlined in the Guide for Care and Use of Laboratory Animals (U.S. National Institutes of Health Publication No. 85-23, revised 1996). All animal experiments were performed in accordance with the relevant institutional approvals (state government LANUV reference number AZ:84-02.04. 2016.A435).

1. Preparation of Instruments, Solutions, and Equipment

1. Prepare 1 L of phosphate buffered saline supplemented with 1 mM magnesium chloride ($MgCl_2$) and 0.1 mM calcium chloride ($CaCl_2$) (PBSCM) and filter it through a sterile filter.
2. Prepare 5 mL of sterile PBSCM per mouse for perfusion and place it on ice.
3. For each mouse, prepare 5 mL of sterile PBSCM supplemented with 0.5 mg/mL biotin.
4. For each mouse, prepare 5 mL of sterile PBSCM and add 0.8 x 10^8 magnetic beads (*e.g.*, 200 µL of a 4 x 10^8 beads/mL solution, without pretreatment) for embolization of the glomeruli. Prepare this solution under the cell culture bench to keep the magnetic beads in the original tube sterile. Place this solution on ice.
5. Prepare a quenching solution by adding 100 mM glycine to PBSCM (5 mL per mouse) and keep it on ice.
6. Make a collagenase solution (0.378 U/mL collagenase A in sterile PBSCM). Per mouse, pipette 1 mL of the collagenase solution into a 2 mL tube and place it on ice.
7. For washing, prepare sterile PBSCM (see step 1.1) in a 50 mL tube and place it on ice.
8. For perfusion, use a syringe pump with a flow of 2.0 mL/min.
9. Prepare a 10 mL syringe with a 21G needle. Place the tip of the needle in a 20-30 cm long catheter (inner diameter, ID = 0.58 mm). Connect the catheter (ID 0.58 mm) with a 10 cm short catheter (ID 0.28 mm) and cut the tip of the smaller catheter oblique for an easy insertion into the mouse aorta.
10. For ligature procedures during the surgery, cut three 5-7 cm silk threads (4-0 to 6-0) per mouse.
11. For the surgery, prepare 3 surgical clamps, 2 surgical scissors, 2 tweezers, 2 fine tweezers, 1 fine scissor, and swabs. Prepare the anesthesia (*e.g.,* intraperitoneal anesthesia ketamine 100 mg/kg bodyweight and xylazine 5 mg/kg bodyweight).
12. For isolation of the glomeruli of two kidneys, use a 100 µm cell strainer, two 50 ml tubes, and a magnet catcher.
13. Prepare CHAPS lysis buffer: 20 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); 20 mM Tris(hydroxymethyl)-aminomethan (Tris) pH 7.5; 50 mM sodiumchorlide (NaCl); 50 mM sodiumfluoride (NaF); 15 mM $Na_4P_2O_7$; 0.1 mM Ethylenediaminetetraacetic acid (EDTA) pH 8.0; 2 mM sodiumorthovanadate; and 2 mM adenosinetriphosphat (ATP).
14. Cool centrifuges to 4 °C.

2. Surgery Under the Microscope

1. Anesthetize the mouse (*e.g.,* with ketamine/xylazine intraperitoneally, see step 1.11). Perform the toe-pinch test to confirm proper anesthesia.
2. Disinfect the ventral side of the mouse with 70% isopropanol.
3. Perform a median cut through the skin from the pelvis to the sternum and remove the skin from the abdominal fascia using tweezers and surgical scissors. Cut the skin on both sides in the middle of the abdomen with surgical scissors.
4. Change surgical tools. Apply a median cut from xiphoid to the bladder through the abdominal muscle layer and divide them into four quadrants using tweezers and surgical scissors. Attach the upper two aside with clamps toward the neck of the mouse with two surgical clamps.
   NOTE: The abdomen is now opened.
5. Put visceral organs aside with a sterile swab and cut the hepatic-phrenic ligament with fine scissors.
6. Free the distal aorta from fascia, fat, and other tissues and prepare a ligation around the hepatic/mesenteric artery cranial of the renal arteries using fine surgical tweezers.
7. Prepare a ligation (with a silk thread 4-0 to 6-0) around the aorta distal of the renal arteries and clamp the vena cava and aorta at the height of the bifurcation.
8. Prepare a ligation (with a silk thread 4-0 to 6-0) around the aorta proximal of the renal arteries on the height of the adrenal gland with two fine tweezers. Tighten the ligation to abolish the blood flow with tweezers.
9. Cut a small hole into the aorta distal to the renal arteries so that the hole is half the diameter of the aorta. Put the catheter into the aorta and fix it with the prepared ligature.
   CAUTION: Avoid bubbles in the perfusion system to prevent air embolism.
10. Start perfusion with the ice-cold PBSCM at a flow rate of 2 mL/min.
11. Cut a hole into the renal vein at the level of the renal arteries with fine surgical scissors and tighten the ligation around the hepatic and mesenteric arteries.
    NOTE: Kidneys should turn pale after starting the perfusion.

3. *In Vivo* Biotinylation

1. Change the syringe bubble-free to ice-cold PBSCM supplemented with 0.5 mg/mL biotin for surface labelling, and perfuse kidneys with 5 mL at a flow rate of 2 mL/min.
   NOTE: Mix the PBSCM solutions (*e.g.,* by turning the 50 mL tubes) gently to avoid forming bubbles within the solution. After aspiration of the solution within the syringe, evacuate any bubbles or air from the syringe. Use an extra cannula (21G) on the syringe to fill the space in the cannula that is connected to the catheter system. Finally, stick the syringe into the cannula with the catheter without bubbles.
2. Change the syringe bubble-free to ice-cold PBSCM supplemented with 100 mM glycine to quench the glomeruli and perfuse with 5 mL at a flow of 2 mL/min.
3. Change the syringe bubble-free to ice-cold PBSCM supplemented with 200 µL magnetic beads/mL and perfuse kidneys at 2 mL/min.
   NOTE: On the kidney surface, embolization of glomeruli with brown magnetic beads will be visible.

4. Isolation of Glomeruli from Two Kidneys

1. Remove kidneys at the hilum and remove the capsule. Place the kidneys on ice in 15 mL of PBSCM in a 10 cm cell culture dish.

2. Cut kidneys into the smallest pieces possible with a new double-edge blade. Move the tissue into a 2 mL tube with 1 mL of collagenase A (see step 1.6) and digest at 37 °C for 30 min. Before and after digestion, mix gently with a 1000 µL cut pipette.
3. Rinse the digested tissue through a 100 µm cell strainer using ice-cold PBSCM. Gently use the cell-scraper to mince the remaining tissue trough the cell strainer and rinse it with ice-cold PBSCM afterwards to a total volume of 50 mL.
4. Centrifuge the suspension at 4 °C 500 x g for 5 min. Remove the supernatant and resuspend the pellet with slightly less than 1.5 mL of ice-cold PBSCM while vortexing the pellet. Afterwards, put the glomerular suspension into a new 2 mL tube.

5. Washing

1. Use the magnet catcher to pull the glomeruli to one side. Wait for 1 min before the glomeruli have moved toward the magnet.
2. Remove the supernatant with a 1000 µL pipette while the 2 mL tube remains in the magnet catcher. During the first and second washing steps (see step 5.3), 250 µL of supernatant remains in the tube to avoid the loss of glomeruli. Perform this procedure quickly to avoid letting tubular structures sink to the bottom of the tube.
   NOTE: The washing procedure will last longer otherwise.
3. Remove the 2 mL tube from magnet catcher and add 1 mL of PBSCM, pipette up and down (very important), then vortex.
4. Put the tube back into the magnet catcher and start over with step 5.2.
5. Repeat the washing steps until the purity of glomeruli has reached 90%. For this, examine a representative aliquot of the supernatant under a microscope (40-100X).
   NOTE: Glomeruli will appear as round structures containing brown magnetic beads. Elongated structures are tubular fragments, and free magnetic beads appear as brown round dots. Cell debris might appear as bulky structures.
6. If purity is reached, estimate the count of glomeruli by dissolving the glomeruli in 1 mL of PBSCM. After mixing, take an aliquot of 10 µL and count the glomeruli under the microscope. Calculate the number of glomeruli with the following equation: final number of glomeruli = number of glomeruli in 10 µL aliquot x 100.
7. Successful isolation of glomeruli leads to a range of 10,000-40,000 glomeruli.

6. Protein Extraction and Immunoprecipitation (IP)

1. Collect glomeruli by centrifugation at 4 °C at 6800 x g for 5 min. Remove the supernatant while using a magnet catcher and resuspend the pellet in ice-cold lysis buffer (*e.g.*, 30,000 glomeruli in 300 µL; CHAPS, see step 1.11). Homogenize the samples with a tissue homogenizer at the highest speed for 30 s and lyse them on ice for 30 min.
2. Remove insoluble material by centrifugation at 15.000 x g for 30 min for 4 °C. Pipette the supernatant which includes the cell lysate into a new 1.5 mL tube. Discard the pellet.
3. Measure the protein concentration of the supernatant by using a bicinchoninic (BCA)-based method following the manufacturer's instructions. A successful lysis of 30,000 glomeruli yields to a glomerular protein amount of 700-1,000 µg/mL. Adjust to equal protein amounts with lysis buffer.
4. Take an aliquot of 10% of the total volume for glomerular lysate and add 2x Laemmli + dithiothreithol (DTT) before incubation for 5 min at 95 °C.
5. For IP, incubate the rest of the lysate with streptavidin agarose beads overnight at 4 °C on an overhead shaker.
6. Centrifuge agarose beads at 1000 x g for 3 min at 4 °C, remove the supernatant, and add 800 µL of lysis buffer to wash the beads for unspecific protein binding.
7. Repeat the wash 3 times, and remove the supernatant completely.
8. Add 30 µL of 2x Laemmli + DTT and incubate at 95 °C for 5 min.
9. Load the lysate and IP probes on a 10% SDS gel and run the SDS gel at 70 V for 30 min, then at 20 mA per gel for 1.5 h. Afterwards, blot the gel for 2h at 200 mA on a nitrocellulose membrane.
10. Block the nitrocellulose membrane overnight at 4 °C with 5% bovine serum albumin (BSA) in washing buffer.
11. Incubate the membrane with the antibody of interest overnight at 4 °C. Wash with washing buffer 3 times for 5 min on a shaker and incubate the membrane with the HRP-tagged secondary antibody for 1 h at room temperature. Repeat the washing step.
12. Visualize the lysate and immunoprecipitation probes on the membrane by using a super-resolution chemiluminescent agent with a CCD camera.

Representative Results

To isolate glomeruli accurately, it is necessary to perfuse murine kidneys with PBSCM first. Perfusion with PBSCM turns kidneys pale (**Figure 1A**). Embolization of glomeruli with magnetic beads will be visible as brown dots on the kidney surface (**Figure 1B**). Isolation of glomeruli with the magnet catcher may show contamination with renal tubuli (**Figure 1C**). Before further analysis of glomeruli, a > 95% purity of glomeruli needs to be achieved by washing the glomeruli more thoroughly (**Figure 1D**).

In vivo biotinylation relies on the ability to label cell surface proteins with biotin. To study this, murine kidneys are perfused with PBSCM or non-cell-membrane permeable biotin. As shown in **Figure 2**, biotin labels the capillary loops in biotin-perfused but not in control mouse kidneys. To investigate cell surface proteins of the glomerulus labeled by *invivo* biotin perfusion, immunoprecipitation of the biotin fraction of glomerular extracts is performed. **Figure 3A** demonstrates that glomerular transmembrane proteins nephrin and podocalyxin are immunoprecipitated within the biotin fraction. However, in control mice, no nephrin or podocalyxin is detected in the immunoprecipitated fraction of biotinylated proteins. As a negative control, the intracellular protein extracellular-signal regulated kinases p42 and p44 (ERK) are not found in the immunoprecipitated fraction of biotinylated proteins of both control and biotin-perfused mouse kidneys. To confirm that the cell surface protein nephrin is actually biotinylated by this method, nephrin is precipitated from control and biotin-perfused mouse kidneys. To visualize biotin, the immunoprecipitated fraction is stained with streptavidin. **Figure 3B** shows biotinylated nephrin in biotin-perfused but not control animals. Lysate controls indicate equal amounts of protein in control and biotin-perfused animals. Endothelial protein vascular endothelial (VE)-cadherin is immunoprecipitated within the biotin fraction as shown in **Figure 3C**. In control mice, no VE-cadherin is precipitated, while VE-cadherin is present in lysates of control and biotin-perfused animals. Intracellular adhesion molecule 2 (ICAM-2) is precipitated from biotin-perfused animals, and no ICAM-2 is found in control animals. Lysates of control and biotin-perfused animals show equal amounts of ICAM-2 (**Figure 3C**). Actin served as the loading control.

This method can be used to quantify amounts of glomerular cell surface proteins in models of nephropathy (*e.g.,* nephrotoxic nephritis, NTN). In the early phase of NTN [day 1 (1 d) after NTN serum injection], proteinuria increases rapidly. In the later phase of NTN [day 18 (18 d)], proteinuria decreases significantly. **Figure 4** shows nephrin cell surface abundance in early NTN (1 d). The *in vivo* biotinylation assay (**Figure 4A**) shows a reduction of cell surface nephrin in NTN animals compared to controls. The densitometric analysis shows a significant reduction of biotinylated nephrin (57%) in NTN animals compared to controls (**Figure 4C**). Quantitative analysis of total nephrin to actin reveals no significant differences between controls and NTN mice (**Figure 4B**). Using a p57 podocyte cell specific staining, podocyte numbers display equal amounts in NTN and control animals (**Figures 4D** and **4E**). **Figure 5** displays the results of nephrin cell surface abundance in late NTN (18 d). **Figure 5A** shows the *in vivo* biotinylation assay in control and NTN mice indicating a recovery of cell surface nephrin. Densitometric analysis displays no significant differences of cell surface nephrin in control and NTN mice (**Figure 5B**). Total nephrin was reduced by 25% in NTN mice (**Figure 5C**). Podocyte numbers were decreased in NTN mice compared to controls by approximately 19% (**Figures 5D** and **5E**).

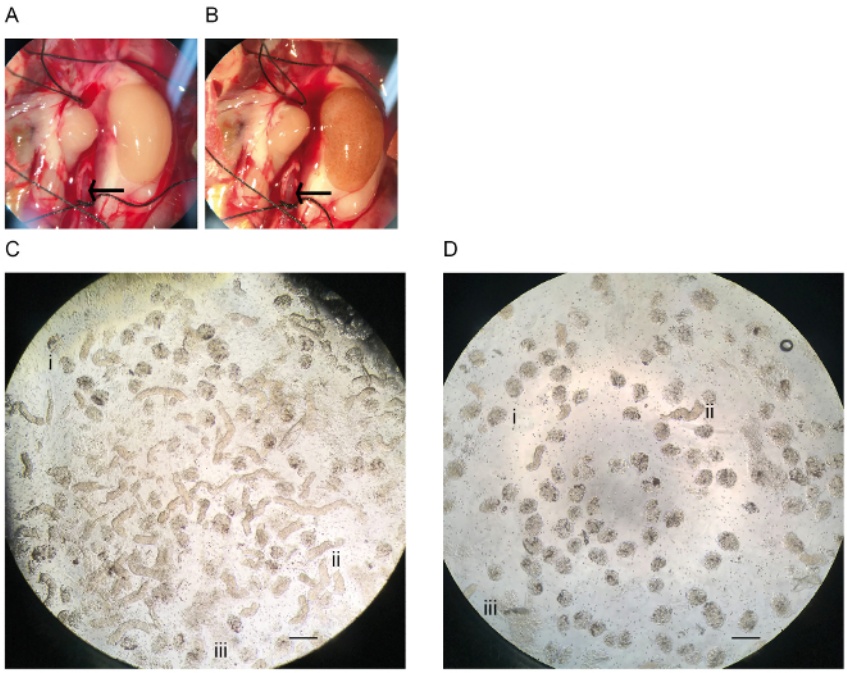

Figure 1: Kidney perfusion and isolation of glomeruli. (A) View through the microscope into the murine situs after perfusion with PBSCM. The catheter in the aorta is indicated with an arrow. Perfusion with PBSCM leads the kidneys to turn pale. (B) Embolization of glomeruli with magnetic beads appear as brown dots on the kidney surface. (C) View though the microscope showing i) contamination of glomeruli (brown round structures); ii) with tubuli (light elongated structures); and iii) cell debris. Purity of the glomeruli is approximately 50%. Free magnetic beads appear as brown dots (magnification 100X). (D) View through the microscope showing a 95% purity of glomeruli (magnification 100X). Scale bars = 100 µm. Please click here to view a larger version of this figure.

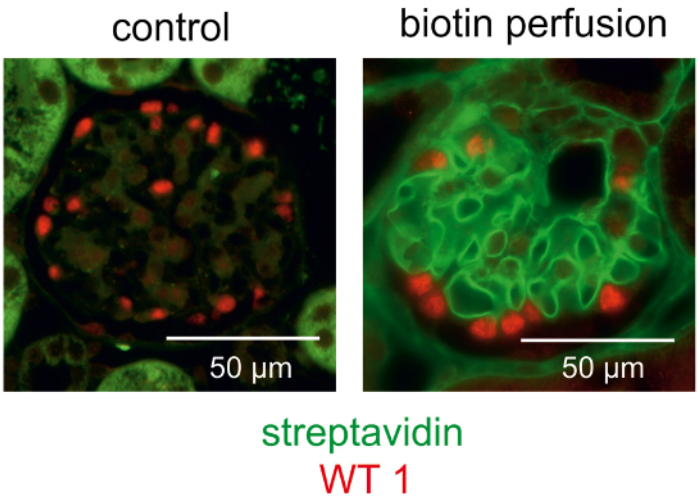

Figure 2: **Biotin detected in the glomerular capillary loop.** Representative immunofluorescence image of mouse glomeruli (C57BL/6) perfused with PBSCM (control) and biotin (biotin). Biotin (green) is visualized by streptavidin in the glomerular capillary loop only in biotin-perfused mice. Control mice do not show glomerular biotin staining. WT1 (red) is detected in the nuclei of podocytes in both mice. This figure has been modified from a previous publication[20]. Please click here to view a larger version of this figure.

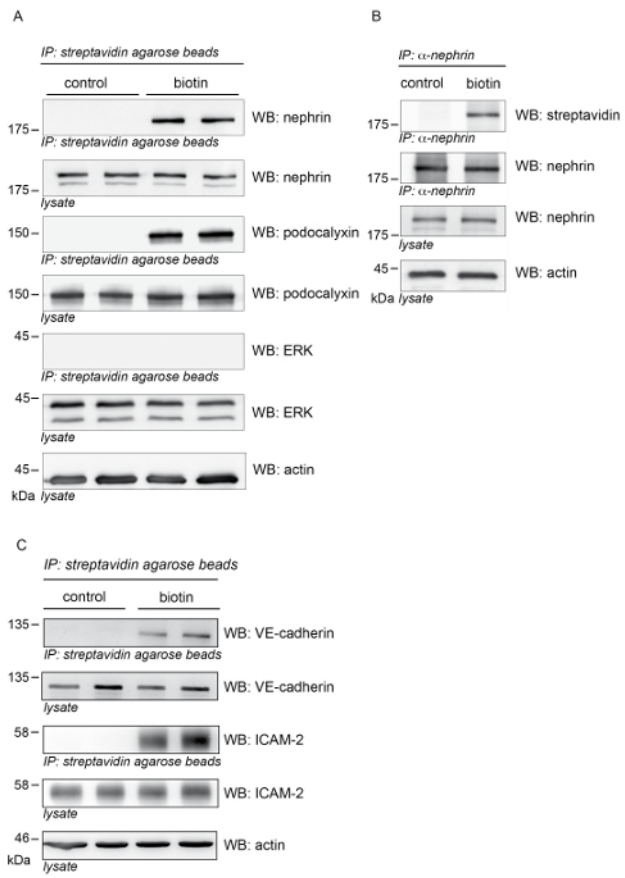

Figure 3: ***In vivo*** **biotin labeling of glomerular cell surface proteins.** (A) Western blot analysis of immunoprecipitated (IP) biotinylated cell surface proteins (IP: streptavidin agarose beads) and lysates (lysate) of kidneys from PBSCM-perfused (control) and biotin-perfused (biotin) mice. The transmembrane proteins nephrin and podocalyxin are only detected in immunoprecipitated samples of biotin-perfused mouse kidneys. In control mice, nephrin and podocalyxin are not detected in immunoprecipitated probes of control mouse kidneys. In lysates of control and biotin-perfused animals, total nephrin and podocalyxin are expressed equally in both groups. The intracellular protein extracellular-signal regulated kinases p42 and p44 (ERK) are not detected in immunoprecipitated probes of control and biotin-perfused mouse kidneys. In the lysates of both mouse groups, ERK is detected in equal amounts. Actin is stained as a loading control. (B) Western blot analysis of immunoprecipitated nephrin (IP: α-nephrin) in PBSCM-perfused (control) and biotin-perfused (biotin) mice. In biotin-perfused kidneys, nephrin is visualized with streptavidin staining, while no detection for nephrin is found in control mice. The immunoprecipitated fraction of nephrin (IP: α-nephrin, WB: nephrin) as well as the lysate fraction (lysate, WB: nephrin) stained for nephrin show equal amounts of nephrin. Actin is used as a loading control. (C) Western blot analysis of immunoprecipitated biotinylated cell surface protein (IP: streptavidin agarose beads) and lysates (lysate) of kidneys from PBSCM perfused (control) and biotin-perfused (biotin) mice. The transmembrane marker protein vascular endothelial (VE)-cadherin and intracellular adhesion molecule 2 (ICAM-2) are only detected in the immunoprecipitated fraction of biotin-perfused animals. In control mice, no VE-cadherin or ICAM-2 are detected. Actin serves as a loading control. This figure has been modified from a previous publication[20]. Please click here to view a larger version of this figure.

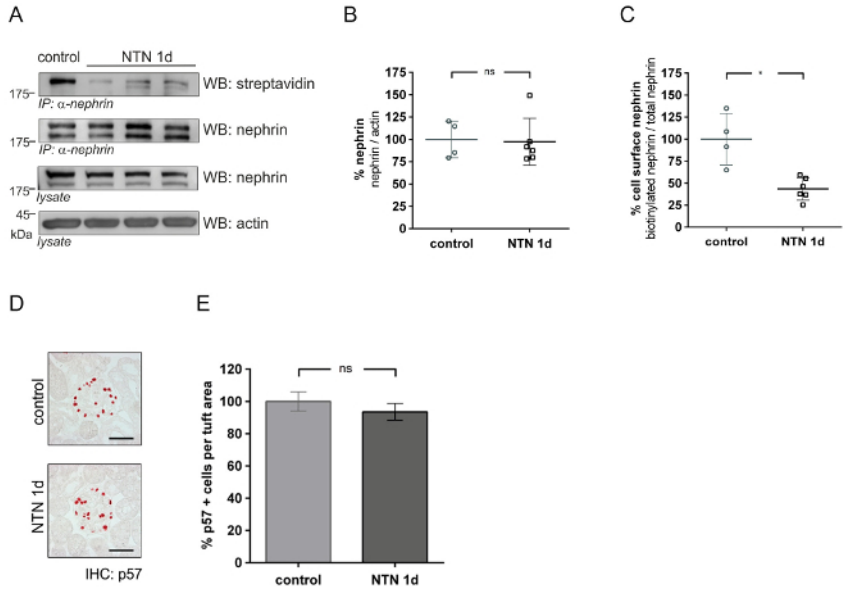

Figure 4: Glomerular protein nephrin abundance in early nephrotoxic nephritis nephropathy (NTN). (A) Western blot analysis of surface nephrin (IP: α-nephrin, WB: streptavidin) and total nephrin (IP: α-nephrin or lysate, WB: nephrin) in control and nephrotoxic serum-treated mice (NTN 1 d). Actin serves as a loading control. Compared to controls, NTN treated mice show reduced cell surface nephrin (IP: α-nephrin, WB streptavidin) compared to controls. (B) Quantitative analysis of total nephrin/actin in control and NTN 1 d mice [control n = 4, NTN n = 6, non-significant differences (ns)]. (C) Densitometric analysis of cell surface nephrin (biotinylated nephrin/total nephrin) in control and NTN mice (*p < 0.01, control n = 4, NTN n = 6). (D) Immunohistochemistry of p57 showing podocytes in control and NTN 1 d mice. (E) Quantitative analysis of p57 positive cells per tuft area (μm^2). (40 glomeruli per mouse quantified, ns). Western blot data show means ± SD. Podocyte counts show means ± SEM. Unpaired *t*-test with Welch's correction. Scale bar = 50 µm. This figure has been modified from a previous publication[20]. Please click here to view a larger version of this figure.

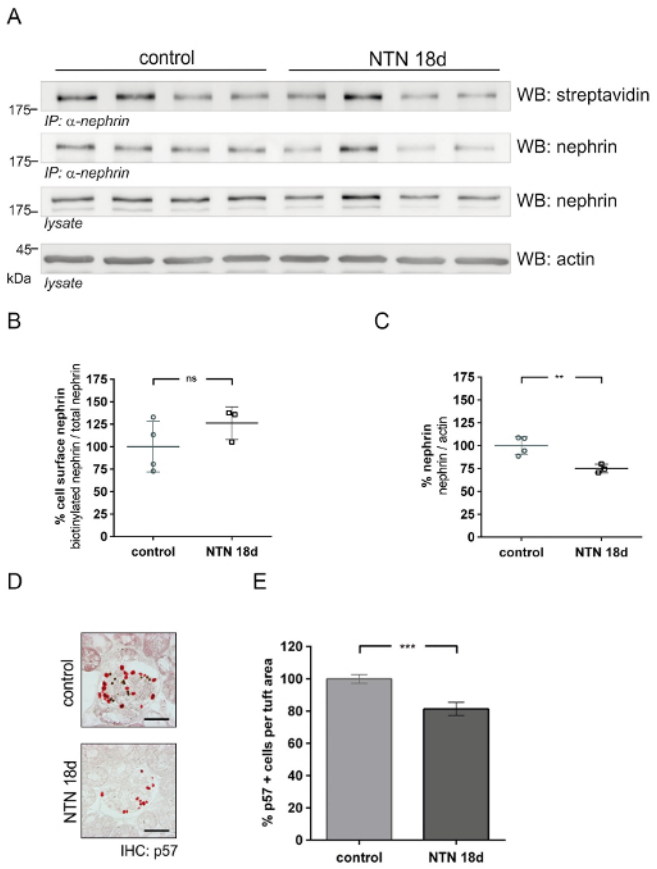

Figure 5: Glomerular protein nephrin abundance in late nephrotoxic nephritis nephropathy (NTN). (A) Western blot analysis of surface nephrin (IP: α-nephrin, WB: streptavidin) and total nephrin (IP: α-nephrin or lysate, WB: nephrin) in control and nephrotoxic serum treated mice (NTN 18 d). In comparison to controls, NTN 18 d mice show equal amounts of cell surface nephrin (IP: α-nephrin, WB: streptavidin). NTN treated mice on day 18 display less total nephrin, and actin serves as a loading control. (B) Densitometric analysis displays no significant differences in cell surface nephrin between controls and NTN 18 d mice (control n = 4, NTN n = 3). (C) Densitometric analysis of total nephrin to actin. In NTN mice at day 18, there is less expression of nephrin compared to controls (control n = 4, NTN n = 3, **p < 0.001). (D) Immunohistochemistry of p57 showing podocytes in control and NTN 18 d mice. There are less p57 positive cells (red) in NTN 18 d mice compared to controls. Magnetic beads appear as black dots. (E) Quantitative analysis of p57 positive cells per glomerular tuft area (μm^2) (***p < 0.0001, control n = 2, NTN n = 2, 40 glomeruli per mouse quantified). Western blot data show means ± SD. Podocyte counts show means ± SEM. Statistical analysis: unpaired *t*-test with Welch's correction. Scale bar = 50 µm. This figure has been modified from a previous publication[20]. Please click here to view a larger version of this figure.

Discussion

The presented method enables successful isolation of glomeruli to investigate glomerular RNA or protein. In addition, primary glomerular cell cultures can be performed from the isolated glomeruli. If biotin is applied before glomerular isolation, labeling of glomerular cell surface proteins can be performed. With this method, *in vivo* glomerular cell surface protein trafficking can be studied, and semi-quantitation of protein abundance is possible. The most critical steps for successfully testing glomerular cell surface protein abundance are 1) developing manual expertise in mouse surgery especially cannulation of the aorta, 2) bubble-free connection of syringes in order to avoid air embolization of the glomeruli, and 3) working under ice-cold conditions once perfusion with PBSCM has started.

For this technique, manual expertise in mouse surgery is essential. Cannulation of the aorta is especially critical, as dissection of the vessel will prevent perfusion of the kidneys. The cut in the aorta should be large enough (approximately 50% of the vessel diameter) to create enough space to introduce the catheter. If the catheter is cut diagonally, introduction of the catheter into the aorta will be easier. In addition to dissection, the introduced catheter should be placed high within the aorta in order to not obstruct the renal arteries. The renal arteries will otherwise not be perfused with biotin and the magnetic beads.

Biotin is a small vitamin that binds with high affinity to streptavidin proteins. Because of its small size (244 Da), biotin does not alter the function of conjugated proteins and will be filtered through the glomerulus. By incubation with streptavidin, biotinylated proteins can easily be separated from untagged proteins by agarose beads or other methods. N-hydroxysuccimide (NHS) esters of biotin bind to amine (-NH2) groups of proteins, which are abundant on side chains of lysine residues, for example. Sulfo-NHS LC-biotin is water soluble and cell-impermeable, if cell membranes are intact. Sulfo-NHS-LC-biotin has been shown to label cell surface proteins[23]. Binding of biotin NHS esters to amine groups is dependent on pH (7-9) and the use of amine-free buffers (*i.e.,* PBSCM). PBSCM with a pH of 7.4 was therefore used to perfuse mouse kidneys with biotin, as it combines ideal physiological properties with optimal solubility and the function of biotin. To quench proteins after labeling,

perfusion of PBSCM with glycine is performed, allowing free biotin to become bound to amine groups of glycine. To prevent cellular processes after death of the animal, it is important to perform perfusion with an ice-cold solution and continue working on ice.

Similar to *ex vivo* biotinylation methods, the mechanical stress of processing glomeruli during *in vivo* biotinylation protocols may also impact endocytosis, rapid signaling events, and RNA integrity. It is therefore essential that all processing steps are performed on ice to reduce the risk of enzymatic cellular activity.

Proteinuric animal models like nephrotoxic serum nephritis (NTN) damage mouse kidneys severely. In particular, NTN results in mesangial expansion, glomerular sclerosis, and tubular lesions, leading to kidney fibrosis in advanced stages of the disease (42 days)[24]. Impaired perfusion of sclerosed glomeruli in disease models may lead to biased results of protein abundance. Sclerosed glomeruli will likely not be perfused with magnetic beads and thus may not become isolated in this method. In disease models leading to severe glomerular sclerosis, using techniques to isolate glomeruli via different sieving steps may be an alternative to the magnetic beads method used in this protocol[25]. However, if glomeruli are severely sclerosed, even perfusion with biotin may be impaired. In addition, *ex vivo* biotinylation, in which extracted glomeruli are bathed *ex vivo* in biotin solutions[21], will probably not be advantageous in these models. Alternatively, use of immunofluorescence to detect protein abundance in severely diseased glomeruli may be advantageous, as it does not rely on perfusion of glomeruli.

Semi-quantitation of protein abundance using this technique works well by using densitometry. However, small differences in protein abundance can be missed as result of the detection limit of densitometry.

The described technique can be transferred to other animal organs that are perfused, provided that the structures of interest are accessible by isolation techniques or the surface protein of interest is specific to one cell type or organ structure. Even though all surface proteins are biotinylated by this technique, using a specific antibody for immunoprecipitation will lead to the fraction of cell surface protein expression of the specific protein (as shown for nephrin in **Figure 3B**).

Disclosures

The authors have nothing to disclose.

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