Video Article

Quantifying Glomerular Permeability of Fluorescent Macromolecules Using 2-Photon Microscopy in Munich Wistar Rats

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URL: http://www.jove.com/video/50052
DOI: doi:10.3791/50052

Keywords: Medicine, Issue 74, Biomedical Engineering, Molecular Biology, Cellular Biology, Anatomy, Physiology, Surgery, Nephrology, Kidney Diseases, Two-photon microscopy, Kidney, Glomerulus, Glomerular Sieving Coefficient (GSC), Permeability, Proximal Tubule, Proteinuria, macromolecules, 2 Photon, microscopy, intravital imaging, munich wistar rat, animal model

Date Published: 4/17/2013

Citation: Sandoval, R.M., Molitoris, B.A. Quantifying Glomerular Permeability of Fluorescent Macromolecules Using 2-Photon Microscopy in Munich Wistar Rats. *J. Vis. Exp.* (74), e50052, doi:10.3791/50052 (2013).

Abstract

Kidney diseases involving urinary loss of large essential macromolecules, such as serum albumin, have long been thought to be caused by alterations in the permeability barrier comprised of podocytes, vascular endothelial cells, and a basement membrane working in unison. Data from our laboratory using intravital 2-photon microscopy revealed a more permeable glomerular filtration barrier (GFB) than previously thought under physiologic conditions, with retrieval of filtered albumin occurring in an early subset of cells called proximal tubule cells (PTC)[1,2,3].

Previous techniques used to study renal filtration and establishing the characteristic of the filtration barrier involved micropuncture of the lumen of these early tubular segments with sampling of the fluid content and analysis[4]. These studies determined albumin concentration in the luminal fluid to be virtually non-existent; corresponding closely to what is normally detected in the urine. However, characterization of dextran polymers with defined sizes by this technique revealed those of a size similar to serum albumin had higher levels in the tubular lumen and urine; suggesting increased permeability[5].

Herein is a detailed outline of the technique used to directly visualize and quantify glomerular fluorescent albumin permeability *in vivo*. This method allows for detection of filtered albumin across the filtration barrier into Bowman's space (the initial chamber of urinary filtration); and also allows quantification of albumin reabsorption by proximal tubules and visualization of subsequent albumin transcytosis[6]. The absence of fluorescent albumin along later tubular segments en route to the bladder highlights the efficiency of the retrieval pathway in the earlier proximal tubule segments. Moreover, when this technique was applied to determine permeability of dextrans having a similar size to albumin virtually identical permeability values were reported[2]. These observations directly support the need to expand the focus of many proteinuric renal diseases to included alterations in proximal tubule cell reclamation.

Video Link

The video component of this article can be found at http://www.jove.com/video/50052/

Protocol

1. Conjugation of Rat Serum Albumin to Sulfo-Rhodamine 101 Sulfonyl Chloride (Texas Red)

1. Dissolve 100 mg of Rat Serum Albumin (RSA) in 6.667 ml of 100 mM Sodium Bicarbonate pH 9.0; final concentration 15 mg/ml in a 50 ml conical tube.
2. Place solution in ice/water beaker and cool to between 0 to 4 °C.
3. Add 200 µl of high quality anhydrous Dimethyl Formamide (DMF) to a 10 mg vial of Texas Red Sulfonyl Chloride (TRSC); vortex on medium for 15 sec.
4. Vortex RSA solution on medium and add the dissolved TRSC.
5. Wrap 50 ml conical in foil (Parafilm can be used to assure a tight seal is formed), place horizontally in ice bucket w/ ice only and place on rocker to agitate solution slowly (avoid formation of bubbles), allow reaction to continue at 0 to 4 °C for 1 hr.
6. Make 5 L of 0.9% saline solution, and wet a 50 kDa molecular weight cut off chamber which can be either a) dialysis membrane with clips, b) dialysis tubing as in a float-a-lyzer, or c) slide-a-lyzer cassette (all suitable for removing unconjugated TRSC).
7. Place reaction mixture in the dialysis chamber and place in the 5 L container w/ the 0.9% saline solution, dialyze overnight at 4 °C with a gentle agitation using a stir bar.
8. Change the 5 L dialysis solution in morning and in late afternoon until an overnight incubation results in virtually no color change (This typically takes ~ 48 hr with at least 4 exchanges). Additionally, with the MWCO of 50 kDa being so close to the MW of RSA, 66kDa; it is

possible to never produce a clear solution; this will be dependent on the distribution in the pore size of the membrane; 60 hr and 5 exchanges is more than sufficient time.
9. Measure volume and divide the initial weight of 100 mg by the volume to give approximate concentration of TR-RSA; typically concentrations range from 10-13 mg/ml. The final dye:albumin ratio should be ~4:1, 1 fluorophore per 15,000 Daltons of protein MW. Store at 4 °C; NEVER FREEZE the TR-RSA solution, as resulting fragmentation that may occur will alter permeability values.

2. Preparing the Inverted Microscope Stage for Imaging/ Microscope Settings

1. Place ~ 4-7 pieces of autoclave tape (approximately ¾" long) perfectly stacked over each other inside a 50 mm dish with a 40 mm coverslip bottom (coverslip bottom dish). These should be placed closer to one of the edges so that the edge of the tape will make contact with the edge of curvature of the kidney but not block the objective light path (**Figures 1A and 1B**); larger rats will require more space between the tape and edge of dish.
2. Place 2 Repti-therm pads next to the stage alongside the 50 mm dish (**Figure 1A**). Place a warming water jacket blanket over the stage.
3. Maximize efficiency when collecting images by assuring the objective turret has a 10x air or 20x (air or water immersion) objective and a higher powered water immersion objective to collect images for quantitation.
4. During imaging the most efficient way to add water to the objectives is to rotate them to the side and add water using a 1 cc syringe with a long piece of PE-200 tubing that can reach the top of the objectives.
5. Set the excitation intensity of the 10watt laser to ~15-20% using the neutral density filters on the software. The gallium-arsenide phosphide non-descanned photodetectors are set to 750 to collect green emissions, and 625 to collect red emissions. Blue emission (such as the nuclear stain Hoechst 33342) are collected using a standard nondescanned multialkaline detector with a setting between 750-800.
6. To assure the proper collection of the low intensity emissions within the Bowman's space, make sure the lower limits of the detectors are set so as not to exclude these values. Visual warning markers will indicate if the sensitivity setting is too low and these values are being given an intensity value of zero.
7. Load a 1 cc syringe with ~ 5-8 mg of the fluorescent albumin solution diluted with 0.9% sterile saline to bring up the total volume to 1 cc.

3. Exposing the Kidney in a Munich Wistar Rat for Intravital 2-Photon Imaging

1. Start with a pre-anesthetized rat using Pentabarbitol (50 mg/ml solution, 120 µl/100 g body weight), Inactin (130 mg/ml solution, 120 µ/100 g body weight), or Isofluorane (5% induction, 1.5 to 2.5% maintenance), an indwelling venous access line (either jugular or femoral venous), and the left flank shaved from just below the ribcage to just above the left thigh.
2. Place the rat flat on its right side so that the left, shaved side is facing up; make sure it is flat on the table, with its posture elongated and not crouched, with front paws touching each other and rear paws touching each other (**Figure 2A**).
3. Very gently palpate to feel the kidney to determine where it naturally lays within the retroperitoneum and draw a straight line parallel to the body (from ribcage to thigh) using a Sharpie (**Figure 2A**).
4. Pick up the skin with a pair of toothed forceps, and pinch the skin along the drawn line using a pair of hemostats to crush the tissue and prevent bleeding. Cut along the incision using a pair of surgical scissors. Crushing the outer skin and the muscle layers prior to cutting will dramatically reduce and typically eliminate bleeding.
5. Repeat this procedure for the outer muscle layer, which is thin.
6. For the incision into the inner muscle layer, which will expose the peritoneum, re-palpate the kidney to estimate size. Pinch an incision line smaller than the kidney; assuring the incision is just over the kidney. It is best to make this incision too small and make it larger if needed. If this is made too big, suturing will be required. This last incision is critical; too far over in any direction will affect stability on the stage and induce either motion artifacts from breathing (best case scenario) or will stretch the renal pedicle and adversely reduce renal perfusion (worst case scenario).
7. Locate the kidney (as shown in **Figure 2B**) and grip the surrounding fat using forceps, working towards the bottom pole of the kidney in a hand over hand fashion.
8. Once the lower pole of the kidney is reached, gently pull the kidney through the incision while very gently squeezing below the kidney to exteriorize. If the incision is too small, crush the muscle layer and cut to widen it; repeat the procedure to exteriorize kidney.

4. Placing the Munich Wistar Rat on the Stage for Imaging

1. Place the kidney towards the edge of the coverslip dish with the kidney slightly rotated towards the dorsal (back side) of the rat so the ventral side of the kidney is making contact with the coverslip bottom dish (**Figure 1A**). Add a warmed, sterile 09.% saline solution to the dish.
2. Look through the 10x or 20x objective and check for motion. If motion is detected, roll the rat over slightly so the thorax is farther away from the coverslip bottom dish; make sure the kidney is as close to the edge of the dish without stretching the renal pedicle (**Figure 1B**).

5. Acquiring Images to Quantify Renal Permeability of Albumin

1. Calculating the glomerular permeability (Glomeruluar Sieving Coefficient; GSC) of any macromolecule will require taking reference background images of individual glomeruli prior to infusion of the fluorescent molecule. If your microscope is equipped with a motorized stage capable of marking locations, find and center individual glomeruli using the lower powered objective and mark each location. A dual pass Fluorescein/Rhodamine epifluorescence filter is ideal for this procedure although either emission filter will work (phase contrast or other non-fluorescence sources of illumination will not work). Glomeruli will appear as empty circular structures surrounded by proximal tubules having an inherent yellow-orange autoflourescence when viewed through the dual pass filter.
2. If your microscope does not have a motorized stage, scan the kidney in a raster pattern with the low power objective and make a rudimentary map of where the individual glomeruli are located; relying on natural landmarks such as large superficial blood vessels located above the renal capsule or fat patches.

3. Switch the turret to the higher power water immersion objective and take 3D data sets of each glomeruli making sure the capillary loops and Bowman's space are clearly visible. Using a pseudocolor palette (something other than B/W) will help to visualize these structures.
4. Focusing in on a superficial blood vessel slowly infuse in the fluorescent albumin making sure time is given to allow for systemic distribution. For molecules with low GSC it is essential to maximize the intensity values in the plasma but not to reach levels that will saturate the photo-detectors in the microscope. This increases detectability of filtered molecules. Note: there is typically a 5-7 sec lapse between the time the fluorescent albumin is introduced to the time it appears on the screen (acquiring full frames at ~ 1 frame/second).
5. Allow approximately 10 min to allow any potential small molecular weight fragments to clear before acquiring 3D volumes at 1 µm intervals to be used in calculating albumin GSC. Typically, the Simonsen's strain of Munich Wistar rats has far fewer surface glomeruli, so all that can be visualized are imaged and quantified. The Frömter strain has a far greater number so we limit the number quantified to ~10.
6. At the end of the study the rat is euthanized via an overdose of the anesthetic used in the study. A dual pneumothoracotamy is performed to insure euthanasia.

6. Calculating GSC for Fluorescent Albumin from 3D Volumes

1. Using Metamorph image processing software load the 3D data sets for each glomeruli, both the background set and set taken after infusion of the fluorescent albumin.
2. In the volume containing the fluorescent albumin locate a superficial capillary loop with enough space empty space between its defined margins and the edge of the Bowman's capsule.
3. In the background volume locate the same focal plane which should contain all the visual cues of the albumin containing image. Select a region within the capillary loop of interest and note the average intensity reading. Next select a region within the Bowman's space and note the average intensity reading. These will be used as background values.
4. For quantitation, select the similar region within the Bowman's space in the albumin containing image. Do this for at least two other regions to take an average value for the average intensity within Bowman's space.
5. Select the capillary loop with the brightest plasma intensity and draw a region around it. Next using the threshold function, highlight the bright values within the circulating plasma, avoiding the dark streaks that are circulating RBC's. Note the average intensity values of the selected plasma space. It is important to preferentially select the bright areas of the plasma because factors within the blood will only serve to cause and underestimation of plasma fluorescence levels.
6. Using a Microsoft Excel spreadsheet enter the values to calculate the GSC where:

$$GSC = \frac{\text{(Raw BowSp intensity-Background BowSp intensity)}}{\text{(Raw CapLoop intensity-Background CapLoop intensity)}}$$

Representative Results

Figure 3 shows an example of an image taken from a surface glomerulus of a Munich Wistar Frömter rat and the steps taken to determine the permeability of fluorescent albumin. The GSC value for albumin of 0.0111 derived for this individual glomerulus fall within the range seen in this strain of Munich Wistar rats when in the fed condition[3]. The stability seen in these images is due to the careful planning and execution of instructions depicted in **Figures 1 and 2**. As stated earlier, the placement of the incision used in exteriorizing the kidney is the most crucial step in producing stable images free of motion artifact.

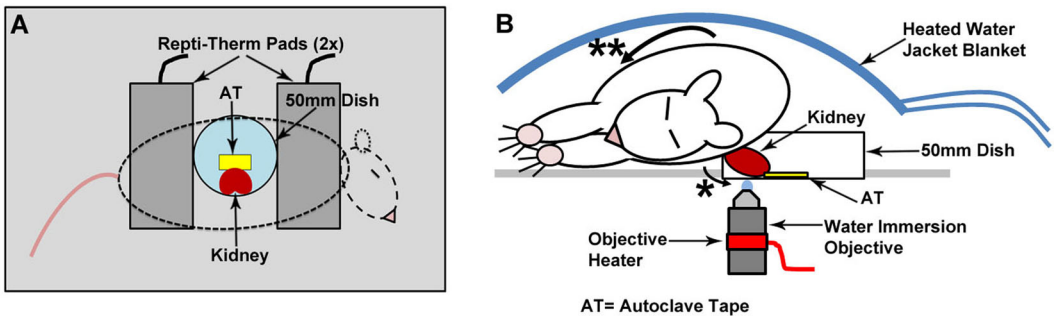


Figure 1. A detailed schematic of positioning an orientation of the rat on the microscope stage prior to imaging. Gently lay the rat kidney side down (as shown in **A**) over the Repti-Therm heating pads with the kidney laying in the coverslip dish. Roll the kidney outward (*) so that the ventral side touches the coverslip bottom and contact is made with the autoclave tape (**AT**). To minimize breathing induced motion, roll the rat forward (**) so that the thorax is out of the area immediately above the coverslip dish. Fill the dish with sterile 0.9% saline and cover with water jacket. Use a thermometer to monitor rectal temperature and turn the Repi-Therm pads on and off as needed. Click here to view larger figure.

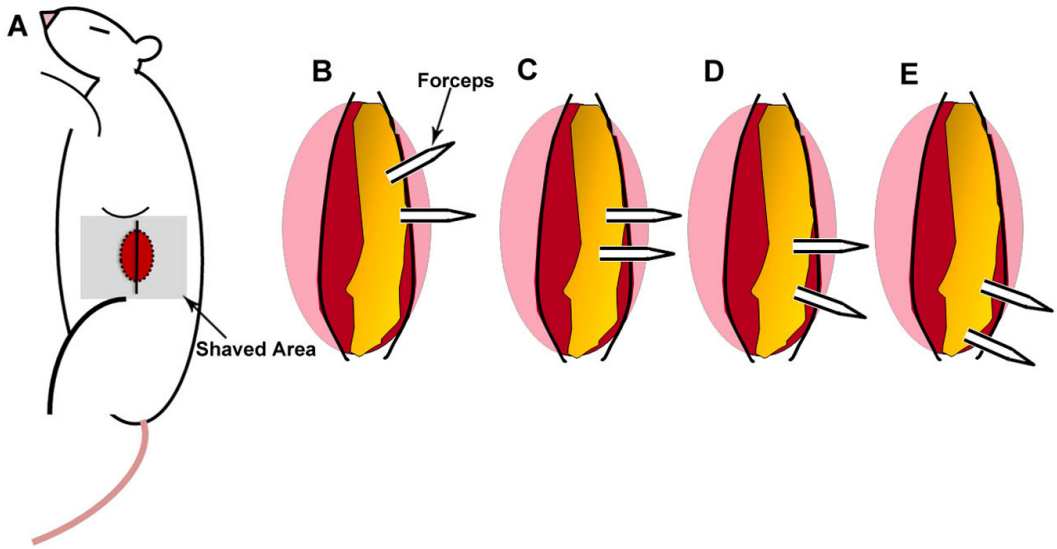
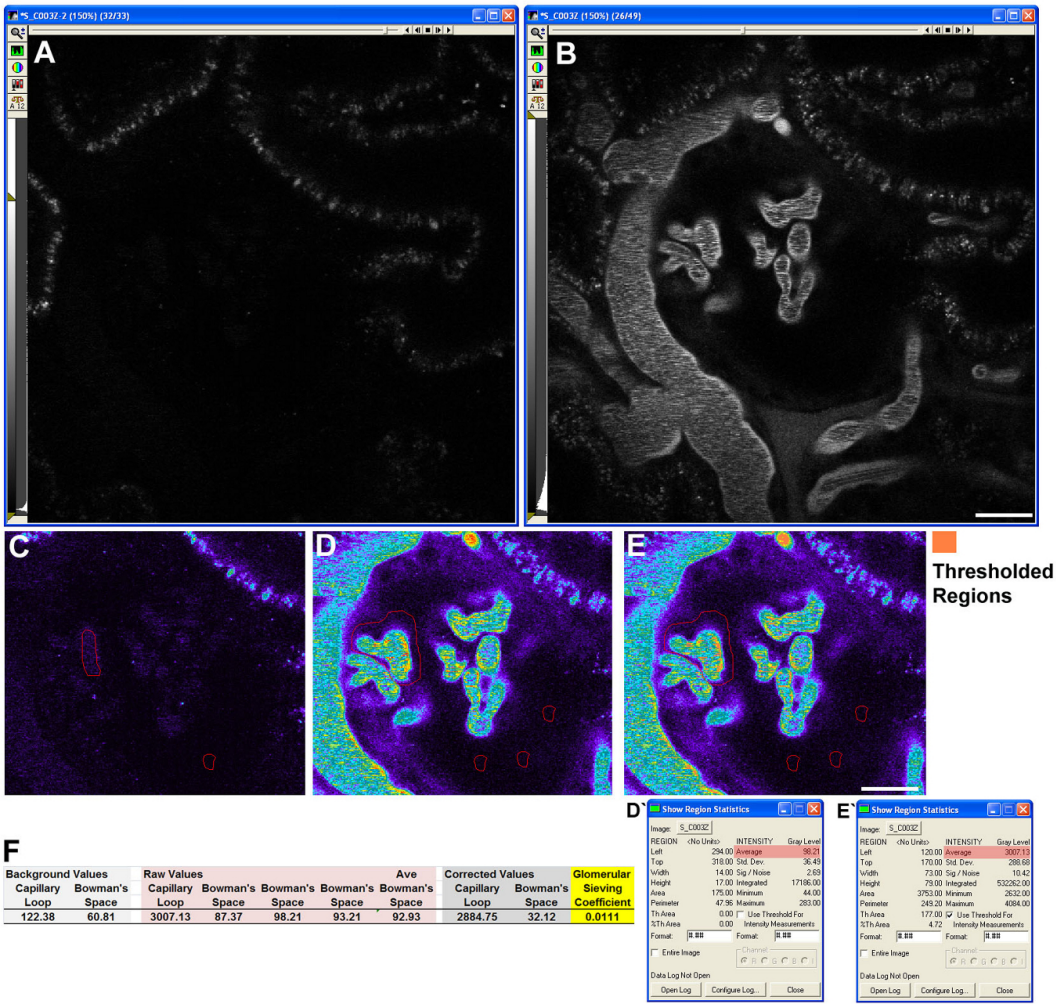


Figure 2. Exteriorization of kidney in Munich Wistar Rat prior to placement on microscope stage. An anesthetized rat is placed with its left size up; area between the rib cage and upper thigh shaved (shown in gray, **A**). Once sequential incisions are made to cut through the skin, outer then inner muscle layer as shown by the line traversing the kidney in **A**. The kidney with associated peri-renal fat should be visible (**B**). Using a pair of forceps, the fat is pinched in a hand-over-hand fashion until the lower most pole is reached (**B-E**). Gently squeeze the area under the kidney while pulling on the fat to exteriorize. Click here to view larger figure.



F

Background Values		Raw Values				Ave	Corrected Values		Glomerular
Capillary Loop	Bowman's Space	Capillary Loop	Bowman's Space	Bowman's Space	Bowman's Space	Bowman's Space	Capillary Loop	Bowman's Space	Sieving Coefficient
122.38	60.81	3007.13	87.37	98.21	93.21	92.93	2884.75	32.12	0.0111

Figure 3. Single plane images taken from 3D volumes showing a glomerulus of a Munich Wistar Rat. Panels **A** & **B** show background images and one taken ~12 min post infusion of Texas Red Rat Serum Albumin (TR-RSA) in black and white. Note the lack discernible intensity of capillary loops (CL) or Bowman's space (BowSp) in the background image (**A**). Panel **C** shows a region taken from panel **A** in pseudocolor to better visualize the low intensity background levels of tissue. Here, a small region is drawn in a capillary loop (longer region) and within Bowman's space to estimate pre-existing fluorescent intensity levels which must be subtracted from values obtained after infusion of fluorescent albumin. Panels **D** and **E** are taken from panel **B** and shown in pseudocolor. Three small regions of interest drawn in Bowman's space are used to calculate the average intensity of fluorescent albumin that has moved across the filtration barrier (**D**). Average intensity values for the individual regions were reported in the highlighted area within the "Show Region Statistics" dialog box (panel **D'**). To calculate the circulating plasma intensity values within capillary loops (CL, in panel **E**) a large region is drawn over the brightest capillary loop and the bright values along the highlighted using a threshold function (shown a orange pixels). Only the intensity values of the pixels highlighted in orange will be reported regardless of the shape or size of the surrounding region. Panel **E'** shows the raw average intensity values of the albumin within the capillary loops; note the checked "Use Threshold For Intensity Measurements" box that is checked. Panel **F** shows the progression of values form left to right starting with the background values for the Capillary Loops and Bowman's space, which are subtracted from the raw values obtained within the images. Once the corrected values are derived, the Bowman's space intensity value is divided by the Capillary Loop intensity value to yield the Glomerular Sieving Coefficient which is a ratio of permeability; impermeant molecules have a value of zero (0), while those that are freely filtered have a value of one (1). Bar = 20 µm. Click here to view larger figure.

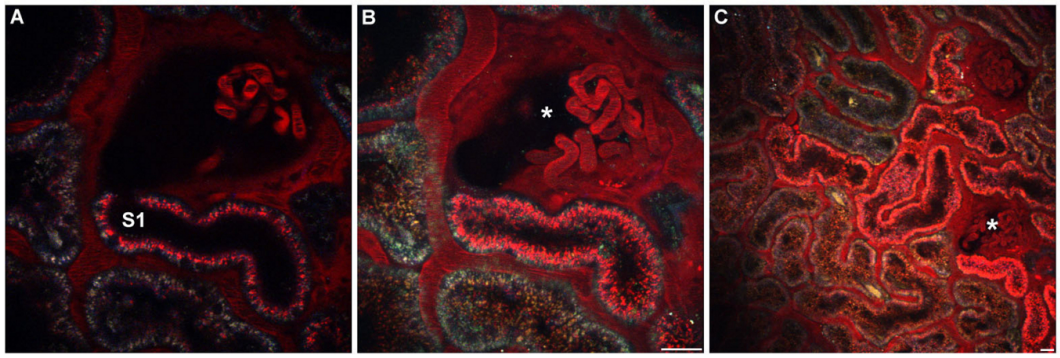


Figure 4. Uptake of filtered fluorescent albumin occurs predominantly in the early segment of proximal tubules, the S1. Panel **A** shows a cross section of a glomerulus and **S1** segment taken ~20 min post infusion of the initial Texas Red RSA bolus. The opening the Bowman's space and avid uptake of the albumin (red) is show in the **S1** segment. Panel **B** shows a shallow 20 µm 3D projection of the same data set. Panel **C** shows a 3D projection using a lower power 20x objective approximately 60 min post infusion. Note the same glomerulus seen in panels **A** & **B** shown in **C** (*) and the higher levels of albumin retrieval seen in that segment versus other proximal tubule segments. Bar = 20 µm. Click here to view larger figure.

Discussion

The steps highlighted here represent what we feel to be ones that will produce consistent and accurate permeability values because they circumvent the following pitfalls:

1. Scattering: The use of a red emitting fluorophores allow for more efficient collection of light since longer wavelength photons are less prone to scatter. Using either green or blue emitting fluorophores will introduce greater variation in GSC's because of the increased variability in intensity values from the capillary loops and Bowman's space[3].
2. Image depth: Although deep 3D data sets are usually collected (often between 30-45 microns in total depth); the upper 10-15 microns represent the most appropriate focal depths to determine GSC values. Again with deeper depths comes a decrease in sensitivity and an increase in variability due to the scatter of emitted photons. More importantly however is the crowding of capillary loops that occurs deeper within the glomerulus. Here, capillary loops are pushed up closely to the edge of the Bowman's capsule surrounding Bowman's space. This increases the chances of inadvertently selecting an area directly over one of the numerous podocytes that surround the capillary loops. This will lead to an underestimation of GSC's because the true, higher intensity of the filtered albumin will not be reported. Note Panel B and D in **Figure 3** and the large amount of space present between the edge of the capillary loops and the edge of Bowman's space[3].
3. Sampling: Selecting several regions within Bowman's space assures the best approximation of detecting filtered albumin levels. It is also important to avoid areas such as the 11 and 12 O'clock position above the capillary loops. Focusing through the 3D volume reveals a set of capillary loops caught almost transversely just below the focal plane. This area would give a falsely elevated GSC value by overestimating the filtered albumin. Conversely, it is important to select the brightest portion of the plasma within capillary loops to best determine the true levels of circulating fluorescent albumin. Here underestimating these levels will also increase GSC by decreasing the value of the denominator in the equation.

Validation of this technique occurs by imaging a freely filtered compound with a GSC of one (1). Here, concerns that consistently underestimating albumin plasma levels, due to optical limitations being responsible for overestimating albumin permeability, are nullified[3]. Additionally, having determined a GSC value for a 70kDa dextran that is virtually identical to that obtained by measuring urinary clearance/plasma levels and micropuncture prove intravital 2-photon microscopy is capable of correctly determining the permeability of fluorescent macromolecules[2]. Finally, the ability to rapidly visualize the glomerulus and tubular segments along the filtrate path, with a high degree of resolution, 2-photon microscopy stands uniquely poised to quantify the importance of the glomerular filtration barrier and PT in determining protienuria and albuminuria.

The protocols described herein are based on the use of a 2-Photon system with an inverted stage which has benefits in the simplicity of the surgical procedures involved and rat placement on the stage. For information on utilizing an 2-Photon system with an upright stage refer to the chapter by Dunn *et al.*[7] in Current Protocols in Cytometry (2007).

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors would like to thank Drs Silvia B Campos-Bilderback and George J Rhodes for completing surgical procedures involving placement of venous access lines. They would also like to thank Sara E Wean for maintaining the Munich-Wistar colonies consisting of both Simonsen and Frömter strains. This work was supported by funding provided to the Indiana Center for Biological Microscopy, and the National Institutes of Health grants P30-DK079312, and 5RO1-DK091623 awarded to Bruce A Molitoris.

References

1. Russo, L.M., *et al.* The normal kidney filters nephritic levels of albumin retrieved by proximal tubule cells; retrieval is disrupted in nephritic states. *Kidney International.* **71**, 504 (2007).
2. Russo, L.M., *et al.* Impaired tubular uptake explains albuminuria in early diabetic nephropathy. *Journal of the American Society of Nephrology.* **20** (3), 489 (2009).
3. Sandoval, R.M., *et al.* Multiple factors influence glomerular albumin permeability in rats. *Journal of the American Society of Nephrology.* **23** (3), 447 (2012).
4. Tojo, A & Endou, H. Intrarenal handling of proteins in rats using fractional micropuncture technique. *American Journal of Physiology.* **263** (4 Pt. 2), F601 (1992).
5. Asgeirsson, D., *et al.* Glomerular sieving of three neutral polysaccharides, polyethylene oxide and bikunin in rat: Effects of molecular size and conformation. *Acta Physiologica.* **191** (3), 237 (2007).
6. Sandoval, R.M. & Molitoris, B.A. Quantifying endocytosis in vivo using intravital two-photon microscopy. *Methods in Molecular Biology.* **440**, 389 (2008).
7. Dunn, K.W., *et al.* Live-animal imaging of renal function by multi-photon microscopy. *Curr. Protoc. Cytom.*, Chapter 12, Unit 12.9 (2007).

