Determination of Podocyte Number and Density in Rodent Glomeruli

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Summary
Reagents and Materials
Protocol

Summary: This protocol describes a validated protocol used by some AMDCC members to determine podocyte numbers in rodent glomeruli.

Reagents and Materials:

Reagent/Material	Quantity Required	Vendor	Stock Number
Universal Imaging Metamorph Imaging System (version 6.1 or later, Molecular Devices) Scientific grade digital color CCD camera (RT SLIDER DIAGNOSTIC). Fluorescent Microscope (Leica DM IRB)			

Protocol:

1) Perfusion fix mouse or rat kidneys with 2% paraformaldehyde or PLP. Embed in paraffin. Cut 3 and 9 um thick sections longitudinally through each kidney (**See Vascular Perfusion Protocol**). It is important to initially establish the actual section thickness generated by each cryotome since this can vary substantially.
2) Stain sections with WT-1 antibody and immunoperoxidase using conventional immunohistochemical procedures and solutions (**See WT-1 Staining Protocol for podocytes for details**).
3) Take photographs of entire cortical area at 40x power using a Spot Advanced Software or similar camera.
4) Photograph 50 consecutive glomerular cross-sections per animal, moving systematically from outer cortex to inner cortex and back again across the slide to ensure equivalent sampling from all cortical regions. Use phase contrast to capture images as this renders WT-1 stained podocyte nuclei a bright golden color that makes them easier to detect.
5) From these photomicrographs (approximately 30/slide), measure the glomerular area (see step #6) by using Metamorph Image Analysis Software and count the podocytes in 50 sequential glomerular cross-sections at two thicknesses (3 and 9 microns).

6) To measure the glomerular area using Metamorph software, camera and microscope should be calibrated and values should be loaded into Metamorph program before outlining the glomerular tuft area. Click on the desktop icon (Metamorph software icon) to open the Metamorph program, and then open the images. From the menu bar, select **Measure**, then select **Calibrate Distance**. A calibration window will appear. Highlight the **40x** calibration and then click **Apply**. Use **Polygon tool** from menu bar for outlining the glomerular tuft area. From the menu bar, select **Measure** and then **Region Measurement** to obtain the measurement of the glomerular tuft area.

7) Some artifacts can appear to be WT-1 positive, but these are usually smaller than podocyte nuclei. To make certain that a reasonable and consistent size cut-off is used to correctly identify podocyte nuclei, choose **Measure** from the menu bar then choose **Manually Count Objects**. Select the number **6**. Use this size restriction method to check if the number 6 from the Metamorph Image Analysis System fits within the nuclear profile. If so, count it. If the number 6 was larger than the WT-1 positive area, do not count it.

Data Analysis:

1) Count the podocyte numbers (P) and measure the glomerular tuft area (GA) from 100 consecutive cross sections per animal (50 each from thick and thin sections) then calculate the average for each set of 50.

2) Divide the average podocyte number (P) by the average glomerular area (GA) to get podocyte number per glomerular area (P/GA) for both 3 and 9 micron sections.

3) The difference between the P/GA of the thick and the P/GA of the thin sections yields the P/GA Δ which is directly related to the actual difference in section thickness (~6 um).

4) Calculate the average glomerular volume per podocyte (GV/P) by dividing the actual section thickness of 6.3 by the P/GAΔ.

5) Determine the glomerular volume by using the Weibel formula
First, calculate the glomerular radius of both thick and thin sections by assuming circular cross sections using formula

$$\text{radius } R = (GA/\pi)^{1/2}$$

and then calculate the average radius (R_{av}) that yielded the average maximum radius

$$R_{max} = 4R_{av}/\pi.$$

Then calculate average glomerular volume as

$$GV = 4/3\pi(R_{max})^3$$

Then divide the average glomerular volume by the average volume per podocyte (GV/P) to get the podocyte number per glomerulus:

$$P = GV/(GV/P)$$

Reference: Sanden SK, Wiggins JE, Goyal M, Riggs LK, and Wiggins RC. Evaluation of a thick and thin section method for estimation of podocyte number, glomerular volume, and glomerular volume per podocyte in rat kidney with Wilms' tumor-1 protein used as a podocyte nuclear marker. *J Am Soc Nephrol* 14: 2484-2493, 2003.