



PODOCYTE COUNTING AND DENSITY ANALYSIS

Version: 1
 Replaced by version:
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Summary: This protocol is used to stain and count podocyte nuclei in mouse glomeruli for diabetic nephropathy study.

Reagents and Materials:

Reagent/Material	Vendor	Stock Number
MACH3 Rabbit HRP-Polymer Detection Kit	Biocare Medical	M3R531L
Da Vinci Green Diluent	Biocare Medical	PD900L
Peroxidase 1	Biocare Medical	PX968H
Betazoid DAB-CHROMOGENK Kit	Biocare Medical	BDB2004L
Tacha's Auto Hematoxylin	Biocare Medical	NM-HEM
Background Snipper	Biocare Medical	BS966
Xylene	Fisher Scientific	X3P-1GAL
Ethanol (EtOH) 200 Proof	Decon-Laboratories Inc	2701
ImmEdge hydrophobic barrier pap pen	Vector Laboratories	H-4000
Humidified chamber	Customized by lab. personnel	N/A
Mounting Medium	Thermo Scientific	8312-4
Cover Glass	Fisher Scientific	12-542-B
PT Link Module	Thermo Scientific	A80400012
Universal Imaging Metamorph Imaging System	Molecular Device	
Scientific grade digital color CCD	RT SLIDER DIAGNOSTIC	N/A

camera		
Microscope and Lense	Leica DM IRB	N/A
Perfusion-fixed paraffin embedded sections	N/A	N/A

Protocol:

WARNING HAZARDOUS CONDITION WARNED AGAINST. This comment describes a hazardous condition to which the technician may be exposed in the performance of this protocol. It also contains directions on how to avoid or minimize the danger. Warnings are always and only used for personnel safety, and precedes the first step that will expose the technician to the hazard.

Protocol 1:

Podocyte Nuclei Staining with Wilms' Tumor-1 (WT-1) Protein:

- 1) Bake slides for 10 minutes at 60°C if it is not baked previously
- 2) Wash 2x in xylene for 5 minutes
- 3) Wash 2x in 100% EtOH for 3 minutes
- 4) Wash 1x in 95% EtOH for 3 minutes
- 5) Wash 1x in 70% EtOH for 3 minutes
- 6) Rinse with dH₂O
- 7) Place in PT Link contain 1x antigen retrieval reagent, pH 9, incubate at 97°C for 20 minutes, and then wait until machine temperature reach to 70°C.
- 8) Wash in TBST for 5 minutes
- 9) Use Pap Pen to encircle tissue
- 10) Block with Peroxidase 1 for 5 minutes
- 11) Wash 2x in 1x TBST for 2 minutes
- 12) Block with Background Sniper for 5 min
- 13) Wash 2x in TBST for 2 minutes
- 14) Dilute WT-1 antibody in Da Vinci Green diluent (1:200)
- 15) Incubate for 2 hours at room temperature in a humidified chamber.
- 16) Wash 2x in TBST for 2 minutes
- 17) Incubate with Mach 3 Rabbit Probe for 20 minutes
- 18) Wash 2x in TBST for 2 minutes
- 19) Incubate with Mach 3 Rabbit HRP Polymer for 20 minutes
- 20) Wash 2x in TBST for 3 minutes

- 21) Develop with Betazoid DAB-CHROMOGENK for 5 minutes, place slide under the microscope to check staining intensity
- 22) Stop reaction in dH₂O
- 23) Counterstain with Tacha's Hematoxylin for 3 minutes
- 24) Rinse in dH₂O to check staining intensity – reapply hematoxylin if not dark enough
- 25) Rinse in TBST to blue
- 26) Wash with dH₂O
- 27) Wash in 70% EtOH for 2 minutes
- 28) Wash in 95% EtOH for 2 minutes
- 29) Wash 2x in 100% EtOH for 2 minutes
- 30) Wash in xylene for 8 minutes
- 31) Mount slides with mounting medium -1 drop
- 32) Insert cover glass carefully, avoid bubble

Protocol 2:

Podocyte Counting and Density Analysis:

Pre-Operating Instructions:

Camera and Microscope should be calibrated and values loaded into MetaMorph® Program.

- 1) Photograph 50 consecutive glomerular cross-sections moving systematically from outer cortex to inner cortex and back so as to provide an equal sample from all cortical regions. Use phase contrast which gives podocyte nuclei golden color that makes them easier to count.
- 2) From these photomicrographs (which contain 1-3 glomeruli per photograph in about 30 photographs), measure the glomerular area by using Metamorph Image Analysis Software (version 6.1) and count the podocytes in 50 sequential glomerular cross-sections at two thicknesses (3 and 9 microns).
- 3) Measure the glomerular area by 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 on **Apply**. Use **Polygon tool** from menu bar for outlining the glomerular tuft area. From the menu bar, select **Measure** and then **Region Measurement** to the measurement of the glomerular tuft area.
- 4) Use a systematic method (large size cut-off) which helps to count WT-1 positive podocyte nuclei but eliminates false counting of granules. From the menu bar, choose **Measure**, then **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 from thick and 50 from thin section) 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 yielded the P/GA Δ which is directly related to the actual difference in section thickness (6.3um).
- 4) Calculate the average glomerular volume per podocyte (GV/P) by dividing the actual section thickness of 6.3 by the P/GA Δ .
- 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:

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

Reagent Preparation:

Reagent 1: 20x TBST Wash Buffer (working concentration 1x)

Reagents and Materials: Tris-base, Sodium chloride, Tween-20, HCl, Distilled water

Procedure:

- Tris-base 48.4g
- Sodium chloride 160g
- Tween-20 10 ml
- Mix to dissolve
- Adjust pH 7.4 with concentrated hydrochloric acid (HCl) before add Tween-20
- Distilled water up to 1L
- Store at room temperature