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U Michigan - Podocyte Counting And Density Analysis V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.565g9g6

Mouse Metabolic Phenotyping Centers
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ABSTRACT

Summary:

This protocol is used to stain and count podocyte nuclei in mouse glomeruli for diabetic nephropathy study.

EXTERNAL LINK

https://mmpc.org/shared/document.aspx?id=322&docType=Protocol

MATERIALS

NAME	CATALOG #	VENDOR
MACH3 Rabbit HRP-Polymer Detection Kit	M3R531L	Biocare Medical
Da Vinchi Green Diluent	PD900L	Biocare Medical
Peroxidase 1	PX968H	Biocare Medical
Betazoid DAB-CHROMOGENK Kit	BDB2004L	Biocare Medical
Tachas Auto Hematoxylin	NM-HEM	Biocare Medical
Background Snipper	BS966	Biocare Medical
Xylene	X3P-1GAL	Fisher Scientific
Ethanol (EtOH) 200 Proof	2701	Decon-Laboratories Inc
ImmEdge hydrophobic barrier pap pen	H-4000	Vector Laboratories
Humidified chamber	Customized by lab. personnel	
Mounting Medium	8312-4	Thermo Scientific
Cover Glass	12-542-B	Fisher Scientific
PT Link Module	A80400012	Thermo Scientific
Universal Imaging Metamorph Imaging System		Molecular Devices
Scientific grade digital color CCD camera		RT SLIDER DIAGNOSTIC
Perfusion-fixed paraffin embedded sections		
Microscope and Lense	Leica DM IRB	Leica Microsystems

MATERIALS TEXT

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

NOTE:

Biocare Medical, RRID: SCR-013549
Thermo Fisher Scientific/ Fisher Scientific, RRID:SCR_008452
Vector Laboratories, RRID:SCR_000821
Vector Laboratories Cat# H-4000, RRID:AB_2336517
Leica Microsystems, RRID:SCR_008960

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

1) Bake slides for 10 minutes at 60ºC 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 dH_2O

7) Place in PT Link contain 1x antigen retrieval reagent, pH 9, incubate at 97ºC for 20 minutes, and then wait until machine temperature reach to 70ºC.

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 dH_2O

23) Counterstain with Tacha's Hematoxylin for 3 minutes

24) Rinse in dH_2O to check staining intensity – reapply hematoxylin if not dark enough

25) Rinse in TBST to blue

26) Wash with dH_2O

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

2 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/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 (Rav) 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:

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