15th International Congress for Stereology and Image Analysis
Aarhus University, 27–30 May, 2019

Abstract

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Determination of Sample Size for Counting Podocytes using the Fractionator/Disector

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Introduction

Kidney diseases affect near half a billion people with few therapeutic options other than kidney transplantation or dialysis. The functional unit of the kidney is the nephron composed of a glomerulus and its tubule (Figure 1). The human kidney has approximately one million glomeruli. The glomerulus is a network of capillaries where impurities in the blood as well as a large amount of water and useful metabolites are filtered across the capillary walls. The filtrate exits the glomerulus and travels through an extensive tubular system (Figure 1) where much of the filtered water and useful metabolites are absorbed resulting in urine.

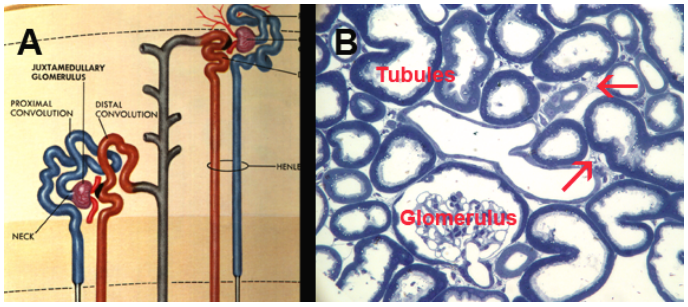

Figure 1: A. 3-D drawing of the outer portion of a kidney showing two spherical glomeruli (in purple) and their associated tubules (in blue and brown). B. 2-D microscopic image of a 1-µ thick section of kidney showing a glomerulus, numerous surrounding tubules and the interstitial space (red arrows) between the tubules.

The glomerulus contains three types of cells. The endothelial cells line the inside of the capillary walls and sit on the glomerular basement membrane. The epithelial cells, known as podocytes, are attached to the glomerular basement membrane on the outside of the capillary walls. The mesangial cells occupy the

space between the glomerular capillaries (Figure 2). The podocyte has been shown to be the primary target of many kidney diseases and the focus of much research during the past decade.

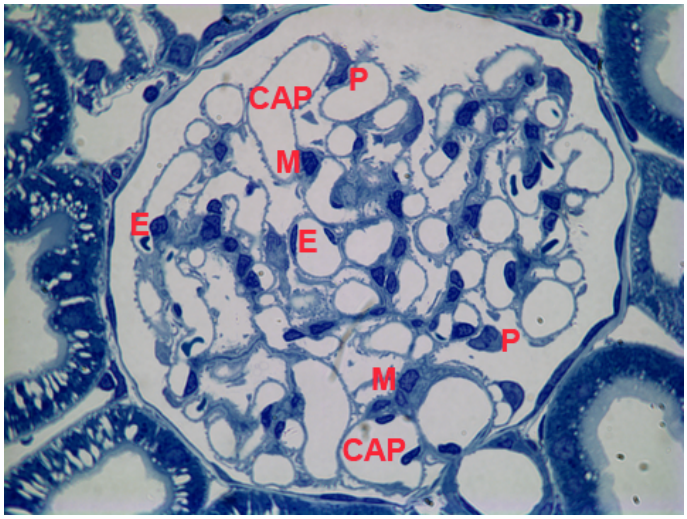

Figure 2: A glomerulus showing capillary lumens (Cap), podocytes (P), endothelial cells (E) and mesangial cells (M).

An early marker of kidney disease is protein in the urine that may indicate the need for a kidney biopsy. A biopsy can confirm disease by showing pathological changes to glomeruli and/or tubules and interstitium. The loss of more than 20% of podocytes may indicate chronic kidney disease leading to complete loss of kidney function [1]. Glomerular sclerosis, tubular atrophy and interstitial fibrosis (scarring) are common endpoints in progressive renal diseases from diverse etiologies. Shroom3 is a protein needed for normal development of several organs including the kidney. Recently it has been shown that a mutation in the Shroom3 gene leads to an overproduction of the collagen protein in the tubule and is associated with loss of kidney function. Surprisingly, patients with the same Shroom3 mutation may be protected from proteinuria suggesting a protective role in the glomerular compartment [2].

To try to understand this dichotomy associated with the Shroom3 mutation, the negative effect on tubule function and a positive effect on glomerular function, mouse experiments were designed to study these effects on glomeruli and tubules. Besides studying the metabolic pathways related to the Shroom3 mutation, structural parameters such as glomerular volume and podocyte number will be studied.

Glomeruli, the cells and the nuclei within the cells are 3-dimensional (3-D) particles but we use 2-dimensional images of these particles to measure structure necessitating the use of stereological tools to carry out the relevant measurements. We will use the Fractionator/Disector [3] tool to count the number of podocytes per glomerulus. Two key questions to be answered before the start of a morphometric study in the kidney are: (1) How many animals are needed per experimental group;

and (2) How many glomeruli per animal need to be measured. To answer the first question a power analysis is required, which will not be discussed here. In this report we will demonstrate how we answered the second question for our future study. Measuring too few glomeruli would result in too imprecise an estimate and measuring too many glomeruli would result in a waste of time and money. In a previous study we used the Fractionator/Disector tool to count podocytes and the data from that study was available for analysis to help determine the optimal number of glomeruli to study per kidney.

Methods

The Fractionator: BALB/c mice age two months were sacrifice and a portion of kidney was fixed in glutaraldehyde and embedded in plastic. An ultramicrotome was used to cut 200 sequential 1-µm thick sections from a tissue block with every 10th section saved to a slide. Thus 1/10 was the fraction of sections from a glomerulus that were saved for analysis. Only glomeruli beginning after the first section of the 200 sequential sections and ending before the 200th section were analyzed. This is the Fractionator principle.

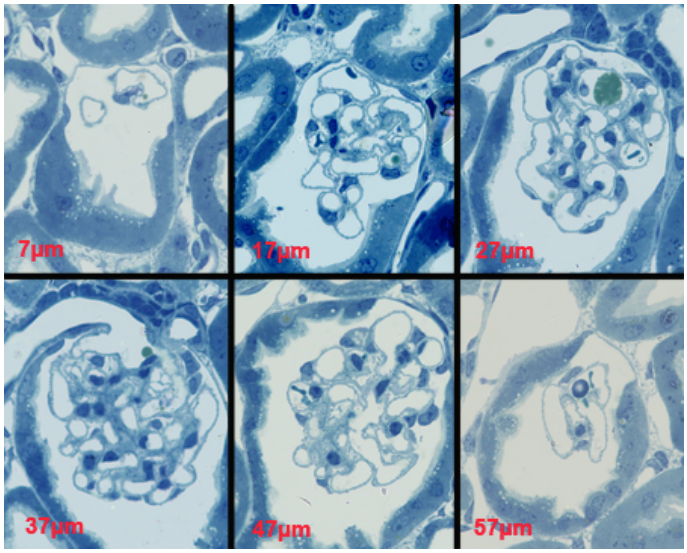

Figure 3: The Fractionator. Six sections through one glomerulus evenly spaced 10 µm apart. The first section is random within the first 10 micrometers of the beginning of the glomerulus. In this example the 7th section was the first saved section. Every subsequent 10th section was saved to a slide resulting in an unbiased systematic sample of the glomerulus.

The Disector: The boundary of podocytes is not resolvable when using the light microscope. Therefore we used the number of podocyte nuclei as a surrogate for the number of podocytes assuming there is one and only nucleus per podocyte. Glomeruli as well as podocyte nuclei are 3-D particles, but in 2-D images we do not see 3-D glomeruli or nuclei, we see 2-D profiles from the glomeruli and nuclei. The number of profiles seen in a 2-D image is not directly related to the number

of particles present in 3-D space. This is known as the Wicksell Problem [4]. The Wicksell Problem was solved with the introduction of the Disector probe by D. C. Stereo [5].

The Disector states that the numerical density of particles in 3-D space can be estimated without bias using a pair of sections, a known distance apart, and counting the number of profiles from particles present in the Reference section but not present in the Look-up section. The numerical density is estimated by the equation:

$$\text{Numerical density} = 1/(d \times \text{Area}) \times \sum Q^{-}$$

where d is the distance between the two sections, Area is the area of the Reference section, and $\sum Q^{-}$ is the sum of profiles counted from nuclei present in the Reference section but not present in the Look-up section.

The Fractionator/Disector: We combined the Disector probe with the Fractionator (Fractionator/Disector) to count podocyte nucleus profiles. When using the Fractionator/Disector one does not need to know the distance between the sections and the number of particles are counted directly not the numerical density as when only using the Disector. To use the Disector two sections were needed. Thus every 10th section was collected (the Reference Section) for the Fractionator and the preceding adjacent section was collected and saved (the Look-up Section). Then profiles from podocyte nuclei seen in the Reference Section but not in the Look-up section were counted (Figure 4).

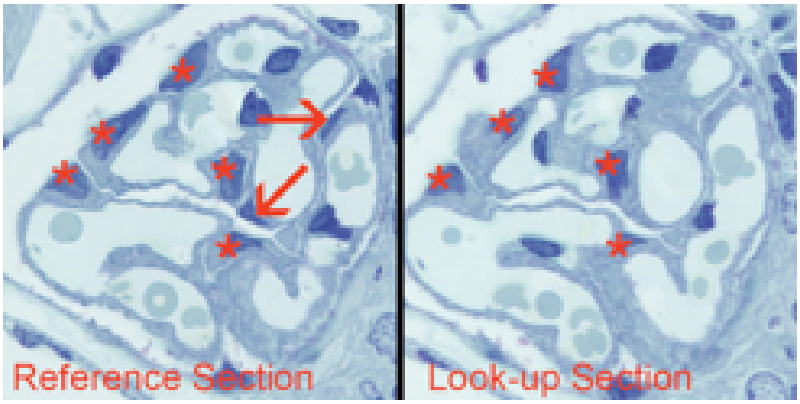

Figure 4: Disector. Profiles of podocyte nuclei present in both sections are marked with a red asterisk and not counted. Two profiles from nuclei in the Reference Section but not present in the Look-up section are counted and labeled 1 and 2.

The estimate of number of podocytes per glomerulus is calculated with the equation:

$$\text{Podocyte number} = 10 \times \sum Q^{-}$$

where 10 is the reciprocal of the fraction of the glomerulus sampled and $\sum Q^{-}$ is the sum of profiles counted from nuclei present in the Reference Section but not present in the Look-up Section.

Results

The number of podocytes counted in each of 7 glomeruli from five animals was available for analysis (Table 1).

After measuring a structural parameter such as podocyte number for several animals in an experimental group, a mean and standard deviation (SD) for the group can be calculated. Dividing the SD by the mean is the coefficient of variation for the group. Within each animal of the group measurements are made in multiple glomeruli and a mean and SD of the parameter for each individual animal can also be calculated. Dividing this within animal SD by the square root of the number of glomeruli measured for the animal is the standard error (SE) for the animal. A coefficient of error (CE) is obtained by dividing the SE by the mean for the animal. This can be repeated for each of the animals in the group (Table 1).

The calculated coefficient of variation for the group is referred to as the observed coefficient of variation (OCV). The OCV can be divided into two components the biological variation (CV) among the animals in the group and the variation among the glomeruli within each animal. If the OCV is perceived to be too large it may be decreased by doing more work (measuring more glomeruli per animal). Before measuring more glomeruli, an expensive task, one can determine if measuring more glomeruli per animal is useful. If most of the variation of the OCV is the biological variation among the animals, measuring more glomeruli per animal will not reduce the OCV very much. However if much of the variation of the OCV is among the glomeruli within each animal, then measuring more glomeruli will reduce the CE and the OCV. The relationship among OCV, CV, and CE is given by:

$$OCV^2 = CV^2 + CE^2$$

We can use the average CE^2 for the animals in the group in the equation. Insert the values for OCV^2 and the average CE^2 into the equation and solve for CV^2. Solve for CV^2. If CV^2 is larger than CE^2, measuring additional glomeruli will not decrease the OCV much, but if CE^2 is much larger than CV^2 then adding glomeruli will decrease CE^2.

For our data the average CE^2 for the five animals was 0.0167, and the CV^2 was 0.0011 (Table 1). CE^2 is much larger than CV^2 meaning the variation among glomeruli within an animal is much greater than the biological variation among the animals. To decrease the CE^2 and move it to more closely match the CV^2, the number of glomeruli per animal should be increased. We double the number of glomeruli per animal, and then repeated the analysis (Table 2). The new average CE^2 was 0.0077 and the CV^2 0.0162 . Now the CE^2 is slightly less than the CV^2 And measuring more glomeruli per animal would not be cost effective.

Table 1

Animnal	Number of Podocyte	Glom 1	Glom 2	Glom 3	Glom 4	Glom 5	Glom 6	Glom 7	Glom 8	Glom 9	Glom 10	Glom 11	Glom 12	Glom 13	Glom 14	Animal Mean	Animal SD	SE	CE	CE^2
1	95	90	140	140	60	60	115	60								95	37	14	0.1466	0.0215
2	99	70	75	115	130	105	90	110								99	22	8	0.0833	0.0069
3	90	88	125	50	155	60	60	90								90	38	15	0.1618	0.0262
4	71	55	115	85	60	100	53	30								71	30	11	0.1587	0.0252
5	79	95	75	70	90	58	78	90								79	13	5	0.0625	0.0039
Group Mean	86.6								OCV^2	=	CV^2	+	CE^2						Average CE^2	0.0167
Group SD	11.6								0.0178	=	CV^2	+	0.0167							
OCV	0.13								0.0178	=	0.0011	+	0.0167							
OCV^2	0.0178																			

Table 2

Animnal	Number of Podocyte	Glom 1	Glom 2	Glom 3	Glom 4	Glom 5	Glom 6	Glom 7	Glom 8	Glom 9	Glom 10	Glom 11	Glom 12	Glom 13	Glom 14	Animal Mean	Animal SD	SE	CE	CE^2
1	100	90	140	140	60	60	115	60	100	150	150	70	70	125	70	100	36	10	0.0956	0.0091
2	104	70	75	115	130	105	90	110	80	85	125	140	115	100	120	104	22	6	0.0555	0.0031
3	85	88	125	50	155	60	60	90	78	115	40	145	50	50	80	85	37	10	0.1176	0.0138
4	74	55	115	85	60	100	53	35	60	120	90	65	105	58	35	74	28	8	0.1021	0.0104
5	77	95	75	70	90	58	78	90	90	70	65	85	53	73	85	77	13	3	0.0448	0.0020
Group Mean	88.0								OCV^2	=	CV^2	+	CE^2						Average CE^2	0.0077
Group SD	13.6								0.0239	=	CV^2	+	0.0077							
OCV	0.15								0.0239	=	0.0162	+	0.0077							
OCV^2	0.0239																			

Conclusion

For our future studies of podocyte number and podometrics using BALB/c mice to study the effects of the Shroom3 mutation and SHROOM3 protein we will use the Fractionator/Disector method and 14 glomeruli per animal.

This report is dedicated to D. C. Sterio on the 35th anniversary of the introduction of the Disector.

References

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