Appendix (Online Supplement)

Detailed descriptions, as well as examples, follow for the Weibel and Gomez method, Disector/Cavalieri Combination, Disector/Fractionator Combination and the Thick-and-Thin Section method.

Weibel and Gomez Method

This method may be used with biopsy tissue processed either for light or for electron microscopy (however, see White[1]). With electron microscopy, podocytes and their nuclei can generally be easily recognized with routine preparation. In light microscopy, either a very well (e.g. perfusion-) fixed specimen with good staining of the glomerular basement membrane must be used, or specific immunostaining (e.g. with WT-1)[2] is needed for identification of podocyte nuclei. Two to four complete section profiles of different glomeruli are chosen randomly (i.e. independently of profile size). The number of podocyte nuclear profiles per unit glomerular cross-sectional area ($N_{Apod,glom}$) and the areal density of these nuclear profiles in the glomerulus ($A_{Apod,glom}$) are determined by, for example, point counting. Calculating an average $N_{Apod,glom}$ and $A_{Apod,glom}$ per subject and using these to calculate an overall $N_{Vpod,glom}$, or calculating an $N_{Vpod,glom}$ for each glomerulus for a single subject and averaging the $N_{Vpod,glom}$ values for the subjects generally makes little difference. The formula used to calculate $N_{Vpod,glom}$ from the measured parameters[3] is:

$$N_{Vpod,glom} = \frac{\kappa}{\beta} \cdot \sqrt{\frac{N^3_{Apod,glom}}{A_{Apod,glom}}}$$

where N_V, N_A and A_A are as above, β is the nuclear shape coefficient (usually values of 1.45-1.65 have been used for rat or human material[4,5]) and κ is the distribution coefficient for variation in the nuclear caliper diameter (usually a value of 1.1 is used, which corresponds to a 20% coefficient of variation in nuclear diameter). The appropriate values for the shape coefficient and the size distribution coefficient should be verified for each experimental situation. To test only for relative differences in podocyte number between two groups, it would be necessary to establish that nuclear shape and size variation are the same in the groups being compared.

Example: Single sections through 3 glomeruli (G1-G3, type 2 diabetes) – using $\beta = 1.45$ and $\kappa = 1.1$

Glomerulus	# of nuclear hits	Sum of nuclear areas (μm^2)	Tuft profile area (μm^2)
G1	17	405.8	29997
G2	24	562.3	34779
G3	2	67.1	2227

From this raw data we can calculate

	$N_{Apo,glom}$ ($1/\mu m^2$)	$A_{Apod,glom}$	$N_{Vpod,glom}$ ($1/\mu m^3$)
G1	17/29997 = 0.00057	405.8/29997 = 0.0135	0.0000880
G2	24/34779 = 0.00069	562.3/34779 = 0.0162	0.0001081
G3	2/2227 = 0.000898	67.1/2227 = 0.0301	0.0001177

The average of the individual $N_{Vpod,glom}$ values is 0.0001046, whereas the 'grand' average $N_{Vpod,glom}$ from the pooled primary measurements of $N_{Apod,glom}$ and $A_{Apod,glom}$ is 0.0000992. Note that the inherent lower weighting accorded to the 'grazing' hit in G3 because of its smaller size results in the grand average being closer to an average of G1 and G2. The grand average $N_{Vpod,glom}$ and average of individual $N_{Vpod,glom}$ values are usually closer to each other than in this example. To obtain the number of podocytes per glomerulus, these $N_{Vpod,glom}$ estimates need to be multiplied by V_{glom}.

Disector/Cavalieri Combination

Podocyte nuclei can be counted using both physical disectors (when pairs of physical sections are used[6,7]) or optical disectors (when optical sections through physical sections are analysed).

When counting podocyte nuclei with physical disectors, the separation of the paired sections must not exceed the average caliper diameter of the podocyte nuclei. Those podocyte nuclei that are hit (sampled) by one of the planes of the physical disector (the reference section) but not by the other (look-up section) are counted (**Figure 2**, main text). The efficiency of the physical disector can be doubled by swapping the roles played by the reference and look-up sections. The combined counts in both directions are then averaged (divided by 2).

When counting podocyte nuclei with optical disectors, thick sections (say at least 14μm) are optically sectioned, either using a brightfield microscope fitted with high numerical aperture objective and condensor lenses, or with confocal microscopy. In both cases, podocyte nuclei are counted at a *unique* moment, such as when they first come into focus, or when they are first seen, or when they disappear (**Figure 3**, main text).

To estimate podocyte number with the disector/Cavalieri combination, the volume of the glomerulus used to count podocyte nuclei must be known. This equals the sum of the areas of all glomerular profiles analysed multiplied by the distance between the paired sections. Multiplying podocyte numerical density by glomerular volume gives absolute podocyte number ($N_{pod,glom}$).

<u>Example</u>: Paired sections through 3 glomeruli (A-C, type 1 diabetes). The distance between paired sections was 60 μm; the reference and look-up sections were 2 μm apart (disector height, h). The efficiency of the disector was doubled by swapping the roles played by the reference and look-up sections. Since in this case podocyte nuclei are in essence counted in each direction (not their unique appearance), the number of unique appearances ($2Q^-$) must be divided by 2, giving Q^-. Glomerular volume was estimated using the Cavalieri principle, with glomerular profile area determined by point counting (40×40 μm grid) in the reference sections. Points landing on the glomerular tuft (P_{glom}) were converted to area by noting the correspondence of one point to 1600 μm^2. Thus, the various calculations are given by:

$N_{Vpod,glom} = Q^- / (h \times A_{glom})$ where h = disector height (2 μm), A_{glom} = glomerular profile area

$A_{glom} = P_{glom} \times \text{area per point} = P_{glom} \times 1600\ \mu m^2$

$V_{glom} = A_{glom} \times t$ where t = distance between sections (60 μm)

$N_{pod,glom} = N_{Vpod,glom} \times V_{glom}$

Glomerulus A			Glomerulus B			Glomerulus C		
	2Q-	P_{glom}		2Q-	P_{glom}		2Q-	P_{glom}
Pair 1	5	15	Pair 1	12	18	Pair 1	6	16
Pair 2	13	23	Pair 2	13	28	Pair 2	15	21
Pair 3	10	17	Pair 3	7	15	Pair 3	5	16
Total	**28**	**55**	**Total**	**32**	**61**	**Total**	**26**	**53**

Glomerulus	$N_{Vpod,glom}$ (1/ μm^3)	Vglom × 10^6 μm^3	$N_{pod,glom}$
A	0.0000795	5.28	420
B	0.0000820	5.86	480
C	0.0000767	5.09	390

Disector/Fractionator Combination

Both the physical disector and the optical disector approach can be combined with a fractionator study design to estimate the total number of podocytes in a glomerulus. For the disector/fractionator method it is not necessary to know either the volume of the disector or the volume of the glomerulus. The number of podocyte nuclei in a known fraction of the glomerulus is counted in each direction ($2Q^-$); multiplying this number by the reciprocal(s) of the sampling fraction(s) gives the total number of podocytes. For estimating podocyte number per individual glomerulus, the whole glomerulus must be sectioned. Whenever an object is completely sectioned, it is important to make a random start of the section series (if every 5th section is to be selected, then one may start with the 1st, 2nd, 3rd, 4th or 5th section overall and continue every 5th section from there on). Podocyte number ($N_{pod,glom}$) is calculated from:

$$N_{pod,glom} = (1/f_1 \times 1/f_2) \times Q^-$$

where f_1 and f_2 are the sampling fractions; for example, when counting podocytes in single glomeruli with the optical disector/fractionator method, f_1 would be the fraction of sections analysed, and f_2 would be the fraction of section thickness analysed per section[8].

<u>Example</u>: Paired sections through 3 glomeruli (G1-G3, type 1 diabetes). Paired 1µm physical sections were taken every 10µm and every 3rd pair was counted, giving sampling fractions of 1/10 and 1/3.

Glomerulus	$2Q^-$	Podocyte number per glomerulus ($N_{pod,glom}$)
G1	34	510
G2	32	480
G3	28	420

Note that with the disector/fractionator combination, podocyte number can be estimated without the intermediate step of estimating podocyte number density ($N_{Vpod,glom}$).
$N_{pod,glom} = 10 \times 3 \times Q^-/2$. Q^- is divided by 2 because podocyte nuclei were counted in both directions using physical disectors.

Thick-and-Thin Section Method

This method may be used with kidney tissue in which multiple sections of at least two different defined section thicknesses (e.g. 3 and 9μm) can be obtained. Unlike the disector, separation of the sections should exceed the caliper diameter of the podocyte nuclei, as multiple hits of the same podocyte nuclei are not desired. The number of podocyte nuclear profiles (hits) per cross-sectional area of reference space ($N_{Apod,glom}$) is related to the number of particles per unit tuft volume ($N_{Vpod,glom}$) *and* to the section thickness. If light microscopy is used, either a very well (perfusion-) fixed specimen with good staining of the glomerular basement membrane must be used, or specific immunostaining (e.g. with WT-1)[2], in order to unambiguously identify podocyte nuclei.

In the classic description by Abercrombie (as applied by Loud and colleagues[9]), $N_{Apod,glom}$ is determined in multiple sections with different section thicknesses. Linear regression of section thickness ***t*** on $N_{Apod,glom}$ gives a slope equal to $1/N_{Vpod,glom}$. In the Thick-and-Thin version of the method, sections of two different thicknesses are obtained and the difference in section thickness ($\Delta \boldsymbol{t}$) between thick and thin sections is divided by the difference in the areal density of podocyte nuclear profiles ($\Delta N_{Apod,glom}$, thick minus thin) to obtain $1/N_{Vpod,glom}$ (often referred to as the glomerular volume per podocyte, or GV/P[2]). Thus,

$$1/N_V = GV/P = \frac{\Delta t}{\Delta N_A}$$

where this form is clearly the 2-point analogue of the above-mentioned linear regression.

Example: Multiple sections (here two) from 2 glomeruli (G1 and G2).

Glomerulus	Section Thickness ***t*** (μm)	#Podocyte Nuclear Profiles	Glomerular profile area (μm^2)
G1	3	7	20,100
	9	18	16,500
G2	3	6	14,000
	9	22	17,300

		$N_{Apod,glom}$ ($1/\mu m^2$)	$\Delta t/\Delta N_A$	$N_{Vpod,glom}$
G1	3	0.00035	-	-
	9	0.00109	6/(0.00109-0.00035)	0.000123
G2	3	0.000428	-	-
	9	0.00127	6/(0.00127-0.000428)	0.000141

References

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