

Diabetic Complications Consortium

Application Title: Serial Block Face Scanning Electron Microscopy Studies of Diabetic Kidney Disease

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1. Project Accomplishments:

1. Tissue collection from Pima Indian persons with type 2 diabetes and control human kidney.
2. Processing the tissues with pre-embedding staining for SBFSEM.
3. Performing SBFSEM on a limited number of specimens
4. Method development for structural quantitative studies
5. Applying the developed methods on SBFSEM images

2. Specific Aims:

Specific Aim 1. Compare 3D models of glomerular compartments in DKD and control kidney tissues.

Results: We have collected and processed kidney biopsies from type 2 diabetic Pima Indian patients as well as control human kidney tissue. These specimens are appropriately processed by pre-embedding staining for SBFSEM. SBFSEM is performed on a limited number of these tissues. 3D modeling workstations are prepared and these images are currently being analyzed.

Specific Aim 2. Compare morphology and quantify number, number density and branching (fission/fusion) of mitochondria in podocytes in DKD and control kidney tissue.

Results: We developed an unbiased size-independent method to estimate mitochondrial number density in podocytes [$Nv(MC/PC)$] and mitochondrial branching reflecting fusion/fission. This method is based on a combination of Euler principle (Kroustrup JP and Gundersen HJG. Estimating the number of complex particles using ConnEuler principle. *J Microsc* 203 (Pt 3): 314-320, 2001) and disector method (Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. *J Microsc* 134 (Pt 2): 127-136, 1984). While the disector method provides an unbiased shape- and size-independent way of counting mitochondria, the Euler principle is used to quantitate mitochondrial branching. Kidney tissue is fixed in 2.5% glutaraldehyde and undergoes pre-embedding staining. SBSEM is performed at about 30,000X with section thickness (z-axis) at 50 nm. An unbiased counting frame is superimposed on the images and sequential paired images are compared to follow mitochondria in to identify convex (islands, *I*), concave (bridges, *B*) and saddle (holes,

H) surfaces within an unbiased counting frame with area A (**Figures 1 & 2**). The Euler number density of MC per PC volume is estimated as:

$$\chi_v(MC/PC) = \frac{(\sum I + \sum H - \sum B)}{2.t.\sum A}$$

Assuming that mitochondria are topologically simply connected with no redundant re-entrant bodies or enclosed cavities:

$$N_v(MC/PC) = \chi_v(MC/PC) = \frac{(\sum I + \sum H - \sum B)}{2.t.\sum A}$$

and the density of MC branching $B_v(MC/PC)$ (equivalent to nodes in topology) per podocyte volume:

$$B_v(MC/PC) = \frac{(\sum B)}{2.t.\sum A}$$

Volume density of MC per PC [Vv(MC/PC)] is estimated using point counting method. A point grid of appropriate density is superimposed in high magnification (30,000x) images Vv(MC/PC) is estimated as the ratio of points falling on mitochondria to those falling on podocyte:

$$V_v(MC/PC) = \frac{\sum P_{MC}}{\sum P_{PC}}$$

The average PC volume is estimated using the point sampled intercept (PSI) method (Najafian et al. PLoS One. 2016 Apr 15;11(4):e0152812). The advantages of this method are: (1) the method is unbiased; (2) it does not require any assumption regarding shape or size of the cell. Podocyte nuclei are identified. A point grid is superimposed on images. A line (intercept line) is passed through each point falling on a podocyte nucleus. Nuclear diameter along the intercept line is measured using Adobe Photoshop measuring tool and according to the PSI method (Figure 3). The average podocyte nuclear volume (\bar{V}_{PCN}) is estimated as

$$\bar{V}_{PCN} = \frac{\pi}{3} (l_{0,0}^3 + 2l_{0,e}^3)$$

where $l_{0,0}$ is the nuclear diameter along the intercept line

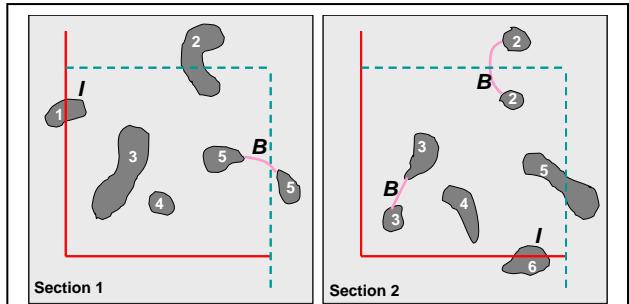


Figure 1. Two sequential images (on z-stack) from an imaginary podocyte with mitochondria cross sections (grey). An unbiased counting frame with inclusion (dashed green) and exclusion (red) lines is superimposed on images. The Euler number in these paired images is determined by comparison of images to identify bridges (B, pink line) and islands (I). There are no holes. MC that cross the exclusion lines (1 and 6) are not counted:

$$\chi = (\sum I + \sum B - \sum H) / 2 = 3/2 = 1.5$$

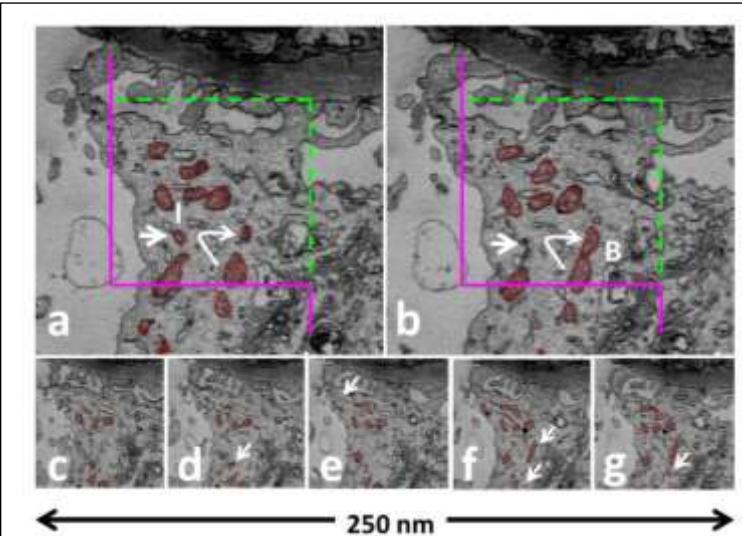


Figure 2. Quantification of mitochondria in a podocyte. (a-b) An unbiased counting frame with inclusion (dashed green) and exclusion (purple) lines is superimposed on images (distance between two adjacent sections = 50 nm). The Euler number in these paired images is determined by comparison of images to identify bridges (B, curved arrow) and islands (I, short arrow). There are no holes. Mitochondria that cross the exclusion lines are not counted.

(red line, Figure 2) passing through the random point (white arrow, Figure 3) and $l_{0,e}^3 = l_{0,i+}^3 - l_{0,i-}^3$, where $l_{0,i+}^3$ and $l_{0,i-}^3$ are distances from the sampling point to the closest and farthest nuclear borders if the intercept line passes through concave regions due to complex shape. In fact, it is not rare to observe more than one nuclear profile in podocyte due to complex shape of their nuclei (Figure 3). Average podocyte volume

$$\bar{V}_{PC} = \frac{\bar{V}_{PCN}}{V_v(PCN/PC)}$$

\bar{V}_{PC} is calculated as $V_v(PCN/PC)$, where $V_v(PCN/PC)$ is the volume fraction of podocyte nuclei per podocyte, estimated by point counting. The average number of mitochondria per podocyte (\bar{N}_{MC}/PC) is calculated:

$$\bar{N}_{MC}/PC = N_v(MC/PC)\bar{V}_{PC}.$$

Total mitochondrial volume per PC ($\sum V_{MC}/PC$) is calculated: $\sum V_{MC}/PC = V_v(MC/PC)\bar{V}_{PC}$

The average mitochondrial volume per podocyte (\bar{V}_{MC}) is calculated:

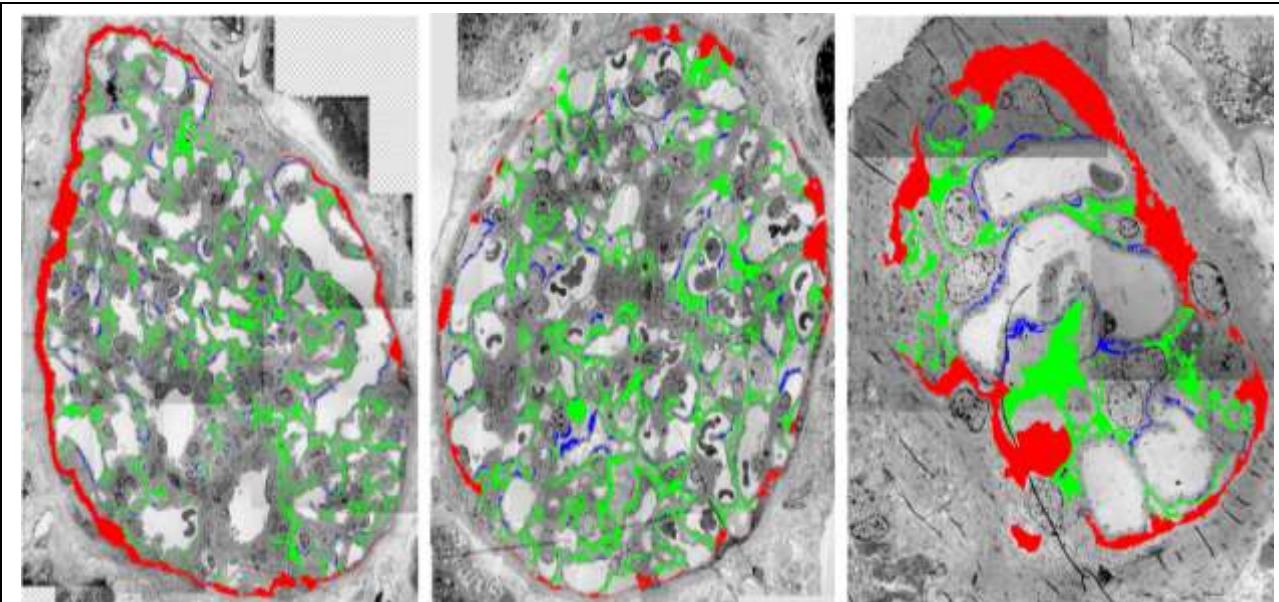
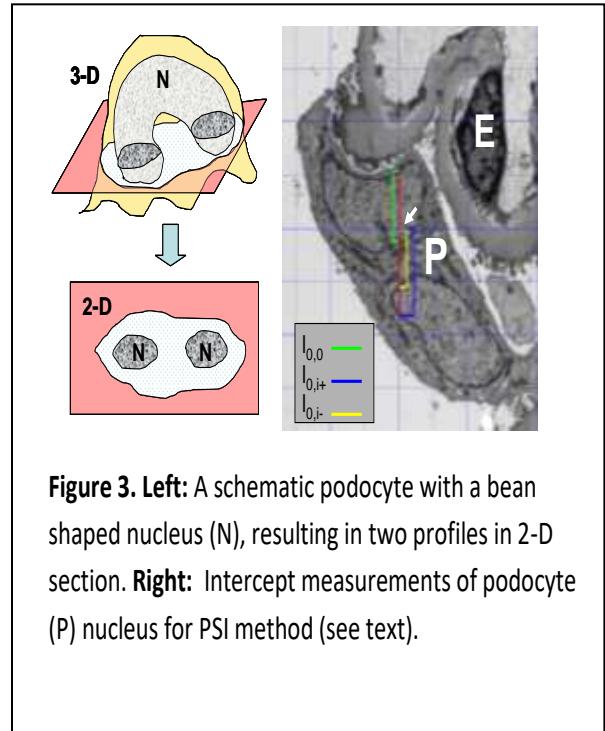
$$\bar{V}_{MC} = V_v(MC/PC)/N_v(MC/PC)$$


Figure 4. Segmentation of SPS (blue), IPS (green) and PUS (red) in three glomeruli. The SPS exit pores are identified as where SPS and IPS (green and blue) spaces meet. The average diameter of exit pores will be estimated using the orthogonal intercept method.

Specific Aim 3. Compare 3D model and size of subpodocyte space (SPS) in DKD and control

kidney tissue.

Results: We prepared montage images of glomeruli from DKD and control kidney tissue. Using a tablet and Adobe Photoshop, we are doing manual segmentation to identify and separate subpodocyte space (SPS), interpodocyte space (IPS) and peripheral urinary space (PUS) (**Figure 4**). Using a line intercept method, total glomerular filtration surface density [$Sv(GBM/glm)$] and filtration surface density for SPS, IPS, PUS are estimated. The SBFSEM z-stack images will be used to confirm the exit pores that are identified on 2 dimensional images. Volume of SPS, IPS and PUS are measured by Adobe photoshop.

3. Publications:

No publications are available at this point.