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Estimation of Nephron Number in Whole Kidney using the Acid Maceration Method

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Phillip Steindel, Ph.D.
Review Editor
Journal of Visualized Experiments

Dear Dr. Steindel,

Please find enclosed our revised manuscript JoVE58599-R1 entitled "*Estimation of Nephron Number in Whole Kidney using the Acid Maceration Method*." We have amended our manuscript to address the minor comments raised by the two reviewers as well as the Editorial comments provided by the Journal's Editorial team. We hope that you now find our manuscript acceptable for publication in *Journal of Visualized Experiments*.

Sincerely,

Sean P. Didion PhD FAHA
Associate Professor

TITLE:

Estimation of Nephron Number in Whole Kidney using the Acid Maceration Method

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KEYWORDS:

Glomerulus, kidney, renal disease, nephron endowment, nephrogenesis, acid maceration

SUMMARY:

Estimates of whole kidney nephron number are important clinically and experimentally, as there is an inverse association between nephron number and an enhanced risk of renal and cardiovascular disease. Herein, the use of the acid maceration method, which provides fast and reliable estimates of whole kidney nephron number, is demonstrated.

ABSTRACT:

Nephron endowment refers to the total number of nephrons an individual is born with, as nephrogenesis in humans is completed by 36 weeks of gestation and no new nephrons are formed post-birth. Nephron number refers to the total number of nephrons measured at any point in time post-birth. Both genetic and environmental factors influence both nephron endowment and number. Understanding how specific genes or factors influence the process of nephrogenesis and nephron loss or demise is important as individuals with lower nephron endowment or number are thought to be at a higher risk of developing renal or cardiovascular disease. Understanding how environmental exposures over the course of a person's lifetime

affects nephron number will also be vital in determining future disease risk. Thus, the ability to assess whole kidney nephron number quickly and reliably is a basic experimental requirement to better understand mechanisms that contribute to or promote nephrogenesis or nephron loss. Here, we describe the acid maceration method for the estimation of whole kidney nephron number based on the procedure described by Damadian, Shawayri, and Bricker, with slight modifications. The acid maceration method provides fast and reliable estimates of nephron number (as assessed by counting glomeruli) that are within 5% of those determined using more advanced, albeit expensive, methods such as magnetic resonance imaging. Moreover, the acid maceration method is an excellent high-throughput method to assess nephron number in large numbers of samples or experimental conditions.

INTRODUCTION:

The nephron is both the basic structural and the functional unit of the kidney[1]. Structurally, the nephron consists of the glomerulus (capillaries and podocytes) located within the Bowman's capsule and the renal tubule, consisting of the proximal tubule, the Loop of Henle, and the distal tubule which terminates into the collecting duct. Functionally, the role of the nephron is the filtration and reabsorption of water and electrolytes and the secretion of wastes. In general, nephrogenesis is completed at 36 weeks of gestation in humans and shortly after birth in several species such as the mouse and the rat[2]. Nephron endowment refers to the total number of nephrons which an individual is born with, whereas nephron number is the total number of nephrons measured at any time post-birth[3]. The term nephron number and glomerular number are often used interchangeably. Because there is only one glomerulus per nephron, the assessment of glomeruli number is an important surrogate for estimating nephron number.

The assessment of nephron endowment and nephron number is of clinical interest as studies have demonstrated an association between nephron endowment and reduced nephron numbers with an increased incidence of cardiovascular disease[4-15]. Based on findings in kidneys at autopsy, Brenner observed that hypertensive individuals presented with a lower total number of nephrons than normotensive individuals[16]. Thus, Brenner hypothesized that there is an inverse relationship between nephron number and the risk of developing hypertension later in life. Brenner also hypothesized that a reduction in nephron number was compensated for by the nephrons that remained. In order to maintain the normal filtration rate in the kidney, residual nephrons compensate by increasing their glomerular surface area (glomerular hypertrophy), thereby working to mitigate any adverse effect of nephron loss on renal function[4,16].

While protective in the short-term, glomerular hypertrophy, in the long-term, leads to increased sodium and fluid retention, increased extracellular fluid volume, and increases in arterial blood pressure, leading to a vicious cycle of further increases in glomerular capillary pressure, glomerular hyperfiltration, and nephron scarring (sclerosis) and injury[4,16].

Obtaining estimates or counts of nephron number offer a couple of experimental advantages: 1) it provides information regarding the process of nephrogenesis, which can then be linked to specific genes or factors in the embryo or maternal-fetal environment, and 2) there is an association of nephron number with cardiovascular disease and, thus, there is the potential that

estimates of nephron number could be used to predict future cardiovascular risk[2,17-22]. In addition to the maternal-fetal environment, several diseases directly impact nephron number and renal function, including atherosclerosis, diabetes, hypertension, and even normal aging[2,9-12,22,23]. Thus, assessment of whole kidney nephron number is important to understand both the genetic and environmental factors that affect nephrogenesis (*i.e.*, nephron endowment) and nephron number over the course of a person's life and the resulting effects on renal function and cardiovascular health.

Currently, there are several methods available for the determination and quantification of nephron number, each with its own advantages and limitations[24-30]. Sophisticated methods for determining whole kidney nephron number include stereological methods, such as the dissector/fractionator method, and magnetic resonance imaging[25,26]. Often considered the gold-standard for determining whole kidney nephron number, the dissector/fractionator method is both expensive and time-consuming. Recent advances and improvement in magnetic resonance imaging and processing have provided the tools to count each and every nephron individually. However, magnetic resonance imaging is not only time-consuming but also extremely expensive. In addition, both the dissector/fractionator method and magnetic resonance imaging requires advanced technical expertise, thus limiting the use of such methods in the majority of research laboratories.

Most methods of determining nephron number make counts or estimates based on the identification of glomeruli, as they are readily identifiable structurally. In this paper, the acid maceration method for estimating nephron number in whole kidney is described and demonstrated[27]. The acid maceration method is fast, reliable, and significantly less expensive than other methods, such as the dissector/fractionator method and magnetic resonance imaging. Moreover, the acid maceration method provides highly repeatable estimates of nephron number that have been reported to be within the range of those determined using magnetic resonance imaging[26].

PROTOCOL:

Supplies and reagents listed below are for the determination of the whole kidney nephron number in one mouse, that is, two kidneys. Modifications for the use of the acid maceration method for rat are identified with asterisks. All experimental protocols conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at The University of Mississippi Medical Center.

1. Kidney Isolation Procedure

1.1. Weigh the mouse (or other species) and euthanize it with an isoflurane (5% - 8%) overdose or pentobarbital (150 mg/kg intraperitoneal injection).

1.2. Once the mouse is euthanized, open its abdominal cavity using fine surgical scissors along the midline.

1.3. Carefully lift the intestines and reproductive adipose to the right side of the abdominal cavity. By gross dissection, isolate the left kidney. Using fine surgical scissors, cut the left renal artery and vein and carefully remove the left kidney, placing the kidney into an appropriately labeled (mouse number/identifier) weigh boat containing phosphor-buffered saline (PBS).

1.4. Repeat the procedure for the right kidney.

1.5. Remove each kidney from its respective weigh boat and place it onto a surgical gauze pre-moistened with PBS.

1.6. Leaving the kidney on the surgical gauze, quickly remove any adherent non-renal tissue (such as perirenal adipose or adrenal gland) followed by the removal of the renal capsule. Weigh each kidney individually, recording the weight of the left and right kidney separately in a laboratory notebook.

2. Homogenization, Incubation, and Straining Procedures

2.1. Once each kidney is weighed, drain each weigh boat of PBS and place the kidneys back into the appropriately labeled weigh boat. Using a clean razor blade, cut the kidney in half, lengthwise. Place each kidney half facing down and cut each half into 2-mm or smaller pieces.

2.2. Using the same razorblade, carefully collect and place the chopped kidney pieces into a labeled 15-mL conical tube (mouse number/identifier; left *versus* right kidney).

2.3. Repeat the procedure for the opposite kidney, using a new razor blade. Place the chopped kidney into a separately labeled 15-mL conical tube.

2.4. In a well-ventilated fume hood, add 5 mL of 6 M hydrochloric (HCl) acid to each 15-mL conical tube.

2.5. Replace the cap to the conical tube, gently agitate the kidney/HCl mixture, and place the 15-mL conical tube into a preheated water bath set at 37 °C for 90 min (*120 min for rat kidneys).

2.6. Briefly agitate each 15-mL tube every 15 min during the incubation in order to ensure that all tissue is exposed to HCl acid.

2.7. Insert an 18-G needle into a 5-mL syringe (*10-mL syringe for rat) and carefully remove the syringe plunger. Place the syringe in a 50-mL conical tube (tube #1) in a fume hood.

2.8. Remove the kidney/HCl solution from the water bath and pour the tissue solution into the open end of the syringe and set the 15-mL conical tube aside in a test tube rack. Carefully replace the plunger and slowly push the plunger so as to extrude the solution through the needle and into tube #1.

2.9. Wash the 15-mL conical tube with 5 mL of PBS solution. Swirl the PBS in the 15-mL conical tube so as to solubilize any remaining kidney tissue.

2.10. Again, carefully remove the plunger from the 5-mL syringe containing the 18-G needle and pour the contents from the 15-mL conical tube into the open end of the syringe. Carefully replace the plunger and flush the syringe by gently pushing down on the plunger, into tube #1. Repeat this process 2x (performed 3x in total).

2.11. Insert a 21-G needle into a new 5-mL syringe (*10-mL syringe for rat) and carefully remove the syringe plunger. Place the syringe with the 21-G needle attached into a new 50-mL conical tube (tube #2).

2.12. Pour the contents from tube #1 into the open end of the syringe containing the 21-G needle. Carefully insert the plunger and flush the syringe by gently pushing down on the syringe plunger and placing the extruded solution into tube #2.

2.13. Wash tube #1 with 5 mL of PBS solution. Swirl the PBS in tube #1 so as to solubilize any remaining kidney tissue.

2.14. Again, carefully remove the plunger from the 5-mL syringe containing the 18-G needle and pour the contents from tube #1 into the open end of the syringe. Carefully replace the plunger and flush the syringe by gently pushing down on the plunger, extruding solution into tube #2. Repeat this process 2x (performed 3x in total).

2.15. Bring the total volume of tube #2 up to 50 mL by adding additional PBS, up to the 50-mL line on tube #2.

2.16. Incubate tube #2 containing the kidney tissue solution in a tube rack on a rocker plate in a refrigerator set at 4 °C overnight (minimum 8 - 10 h).

3. Counting of Glomeruli and Extrapolation of the Nephron Number

3.1. Remove tube #2 from the refrigerator and resuspend the pelleted tissue by gently inverting the tube several times in order to create a homogenous solution. We recommend counting glomeruli within 5 d after processing.

3.2. Carefully aliquot 500 µL of the kidney solution into a single well of a 12-well plate. Repeat this 2x, placing each aliquot into a separate well so that there are three wells of kidney solution per kidney, for analysis in triplicate.

3.3. Add 500 µL of PBS to each of the three wells containing the kidney solution, for a 1:1 dilution.

3.4. Using an inverted microscope, count the number of glomeruli per well. Counting is aided by using a grid of 16 separate sections placed on the bottom of each well. Count the number of

glomeruli per each gridded section and then sum the count per grid to get the total number of glomeruli per well. Glomeruli are readily identifiable by their spherical structure. Additional identifiers included a reddish hue due to blood-filled capillaries, as well as pre- or post-arterioles that remain attached to the body of individual glomeruli (**Figure 1**).

3.5. Add the total number of glomeruli counted per each of the three wells and then divide them by three for the average number of glomeruli per 500 µL of kidney solution. If the variance in the average number of glomeruli per well is greater than 10%, repeat the nephron-counting procedure, paying close attention to the homogeneous nature of the kidney solution. Multiply the number of glomeruli counted per well times 100 for the average number of glomeruli per kidney. Total nephron number can be expressed per kidney or, using kidney weight, per mg or g of tissue.

REPRESENTATIVE RESULTS:

Below are representative estimates of whole kidney nephron number from an established mouse model of hypertension and a genetic rat model of age-related chronic kidney disease. Key identifying characteristics of glomeruli, such as a spherical structure with or without attached pre- or post-arteriolar or tubular structures, are highlighted for those new to the acid maceration method (**Figure 1**).

In the first experimental example, total nephron numbers were determined in mice (male C57BL/6, 6 weeks of age) infused with vehicle (saline) or angiotensin II for 14 days. In vehicle-infused mice, nephron number was 12,411 ± 248 nephrons per kidney as determined using the acid maceration method (**Figure 2**). These estimates are consistent with ranges of whole kidney nephron number reported previously in mice (**Table 1**). Angiotensin increased atrial pressure by approximately 40 mmHg following 14 days of infusion and was associated with a significant reduction in nephron number (9,122 ± 193 nephrons per kidney) of nearly 26% (**Figure 2**). These data suggest that angiotensin II infusion is not only associated with hypertension but, also, a significant reduction in nephron number is associated with nephron loss due to sclerosis or glomerular injury.

In the second experimental example, previous findings of nephron number in a genetic rat model of renal agenesis, the heterogeneous stock-derived model of unilateral renal agenesis (HSRA) rat, were confirmed. The HSRA model exhibits incomplete penetrance phenotypes for defects of the kidney and urinary tract, with some animals being born normal (with two kidneys) and others born with one kidney. In rats born with two kidneys (male hA-Control; 12 weeks of age), estimates of nephron number using the acid maceration method were 27,288 ± 336 nephrons per kidney (**Figure 3**). In contrast, in rats born with a solitary kidney (male hA-Solitary; 4 weeks of age) estimates of nephron number were found to be significantly lower at 24,594 ± 883 nephrons per kidney (**Figure 3**). Estimates of nephron number attained in these samples are also consistent with ranges previously reported in the rat (**Table 1**). Note that these data are consistent with previous findings in which hA-Solitary rats were found to exhibit decreased nephron endowment or numbers (compared to one control kidney) most likely reflective of the underlying genetic factors associated with being born with a single kidney[31].

FIGURE AND TABLE LEGENDS:

Figure 1: Key identifiers of glomeruli for counting and assessing whole kidney nephron number. Glomeruli are readily identifiable by their spherical structure, as indicated by the arrows. Additional identifiers include a reddish hue due to blood-filled capillaries, as well as pre- or post-arterioles (or tubules) that may remain attached to the body of individual glomeruli following processing (like the one identified at the bottom of this micrograph). Also present in this image are remnants of tubules and blood vessels. Magnification = 10X; scale bar = 100 µm.

Figure 2: Nephron counting in mice. (**A**) Representative image of glomeruli from a single mouse kidney plated in a 22.1-mm flat-bottomed well of a 12-well culture plate, as seen using an inverted microscope. Glomeruli and renal tubules are seen at moderate density when diluted by a factor of two (0.5 mL of kidney solution plus 0.5 mL of 1x PBS). (**B**) Effect of a 14-day vehicle or angiotensin II infusion on nephron number in male C57Bl/6 mice as assessed by glomerular counting. The angiotensin II infusion at 1,000 ng/kg/min (*via* osmotic minipump) was associated with hypertension and a marked a reduction in nephron number as compared to the nephron number in mice infused with vehicle. Nephron numbers in vehicle-infused mice are consistent with previously reported estimates of nephron number in mice (**Table 1**). Vehicle: $n = 3$, angiotensin II: $n = 3$, $^*p < 0.05$. Error bars = SE. Magnification = 4X; scale bar = 250 µm.

Figure 3: Nephron counting in rats. (**A**) Representative image of glomeruli from a single rat kidney plated in a 22.1-mm flat-bottomed well of a 12-well culture plate, as seen using an inverted microscope. Glomeruli and renal tubules are seen at a significantly higher density as compared to that in the mouse. (**B**) Quantification of the nephron number in HSRA-Control and HSRA-S rats reveals that nephron number is less in HSRA-S as compared to HSRA-C rats and consistent with previous findings in this model[31]. HSRA-C, rats born with two kidneys: $n = 3$; HSRA-S, rats born with a solitary kidney: $n = 3$, $^*p < 0.05$. Error bars = SE. Magnification = 4X; scale bar = 250 µm.

Table 1: Reported ranges of nephron number in several species.

DISCUSSION:

With good experimental technique, the acid maceration method is ideal for estimating nephron number in whole kidney. Although the kidney is dissolved in acid, glomeruli remain largely intact and are readily identifiable, making the counting of individual glomeruli relatively easy and straightforward. The acid maceration technique is particularly advantageous for several reasons. First, the acid maceration method is a rapid and convenient method that requires relatively little in terms of expense and physical effort. All reagents and consumables required to perform the acid maceration method are readily available in most basic laboratories, the only major requirement being access to an inverted microscope for glomerular counting. In terms of expense, it is estimated that the acid maceration method costs just a couple of hundred dollars per animal, which is not a lot when compared to costs associated with other methods of whole

kidney glomerular counting, such as magnetic resonance imaging, which can range into thousands of dollars per animal of scanning time and technical expertise.

Second, the acid maceration method involves significantly less tissue-processing time compared to other methods of whole kidney nephron number such as the dissector/fractionator method[24]. From start to finish, the acid maceration method requires less than 24 hours of total time, of which less than 1 - 2 hours is spent processing kidneys and then counting the individual glomeruli per experimental animal. In contrast, the dissector/fractionator method is labor-intensive, requiring an estimated 15 hours of labor (4 - 6 hours of sectioning, 2 - 3 hours of staining, and 4 - 5 hours of counting time) per kidney, not including the 48 - 72 hours needed to process the kidneys in glycol methacrylate[24]. Because of the advantages of cost and time, the acid maceration method is especially useful in studies where estimates of whole kidney nephron number need to be made in large numbers of animals with or without genetic or pharmacological interventions. Both the cost and time involved with the dissector/fractionator method and magnetic resonance imaging have been considered a major limiting factor in their adoption in most research laboratories.

Finally, the acid maceration method provides estimates of whole kidney nephron number that are comparable to measures using more sophisticated methods such as magnetic resonance imaging[26]. For example, using cationic ferritin-labeling of glomeruli and three-dimensional image processing to count every nephron in a single kidney from the Sprague-Dawley rat, magnetic resonance imaging yielded on average counts of 34,000 individual glomeruli per kidney[26]. In non-cationic ferritin-labeled kidneys, magnetic resonance imaging yielded counts of 2,000 nephron-like structures due to the counting of non-glomerular regions of kidney with magnetic signals of similar magnitude and shape as ferritin-labeled glomeruli. When these artifacts are subtracted from the counts determined by cationic ferritin-labeled kidneys, the effective number of glomeruli determined, using magnetic resonance imaging, is closer to 32,000 nephrons per kidney[26]. In this same study, validation experiments yielded average glomerular counts of 31,000 per kidney using the acid maceration method[26]. Thus, the acid maceration method produces estimates of whole kidney nephron number that are within < 5% of those determined using state-of-the-art techniques such as magnetic resonance imaging.

Although there are several key advantages associated with the acid maceration method, investigators should also be aware of the limitations associated with this method. One limitation relates to the use of whole kidney. As the whole kidney is dissolved and homogenized, information regarding the spatial distribution of glomeruli within the kidney cortex cannot be determined. If knowing the intrarenal distribution of glomerular volume is important, then the use of magnetic resonance imaging would be a better-suited method.

Compared to other methods, the use of the acid maceration method appears to slightly underestimate total nephron number. The smaller estimates are likely due, in part, to the dissolution of a small percentage of glomeruli in the acid or during the maceration of the kidney. In addition, with aging or disease, there is a loss of functional nephrons, which, though not contributing to urine formation, may be more sensitive to acid maceration and may be destroyed

during processing, thus contributing to lower estimates of total nephron number. Thus, care should be used when collecting and processing kidneys so as not to leave any kidney tissue *in vivo* when chopping the kidney, or in the syringes when extruding tissue samples. Overall, however, the underestimation of total nephron number due to tissue processing appears to be relatively small.

As the counting of glomeruli using the acid maceration method is subjective, the underestimation of nephron number may also be reflective of observer bias, which can be easily overcome by measurements being performed by two separate investigators blinded to information regarding experimental animal or condition. If there is a significant concern as to what constitutes a glomerulus, an alternative approach would involve infusing experimental animals with iron oxide before euthanasia. When infused intravenously, iron oxide will enter and become trapped in the glomerulus; thus, processed glomeruli should stain dark black, allowing for a greater identification of glomeruli when counting[30].

The acid maceration method estimates whole kidney nephron number based on the measurement of small samples (3 x 0.5 mL or 1.5 mL) as opposed to measurements of the entire solution of kidney (50 mL). The acid maceration method is highly reproducible in nature, and glomeruli counts within samples of a single kidney and within experimental groups are found to be highly consistent. Moreover, representative results reported in the present study from wild-type C57Bl/6 mice and rats born with a single kidney (**Figures 2** and **3**) are highly consistent with previous findings, as well as with ranges previously reported (**Table 1**)[5,31]. If desired, additional measurements could be easily made to better estimate the total nephron number per kidney.

While the acid maceration technique provides reliable and repeatable estimates of nephron number, this method is not recommended for obtaining measurements of glomerular volume, as exposure to acid most likely produces alterations in glomeruli volume. As the determination of glomerular volume is important in determining the whole kidney glomerular surface area, it is recommended that such measurements be made using histological and stereological methods[24,25]. Particularly, stereological methods that make use of glycol methacrylate embed kidneys in order to limit tissue swelling and expansion and, thus, maintain glomerular geometry as close as possible to that *in vivo*.

Finally, another limitation of the acid maceration technique, and one that is common to other methods of assessing nephron number, is that no information regarding glomerular function (such as glomerular filtration rate) can be ascertained from glomeruli once processed. Thus, while individual glomeruli can be counted, its functional status (urine-forming or non-urine-forming) cannot be determined using the acid maceration method. Similarly, no longitudinal information can be ascertained using the acid maceration method in individual mice. Instead, separate groups of animals at various time points are required to gain insight into changes in nephron number over an animal's lifespan or pre- or postgenetic or pharmacological intervention.

In summary, the acid maceration method is a low-cost, high-throughput, and efficient method to estimate whole kidney nephron number. The acid maceration method provides a high degree of

reproducibility as evidenced by the two examples presented here, as well as by those previously reported using this method[18-22,28]. While the use of the acid maceration method was described for use in the mouse (and rat), the acid maceration method can also be scaled for use in larger species, such as the dog and pig[27,32].

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DISCLOSURES:

The authors have nothing to disclose.

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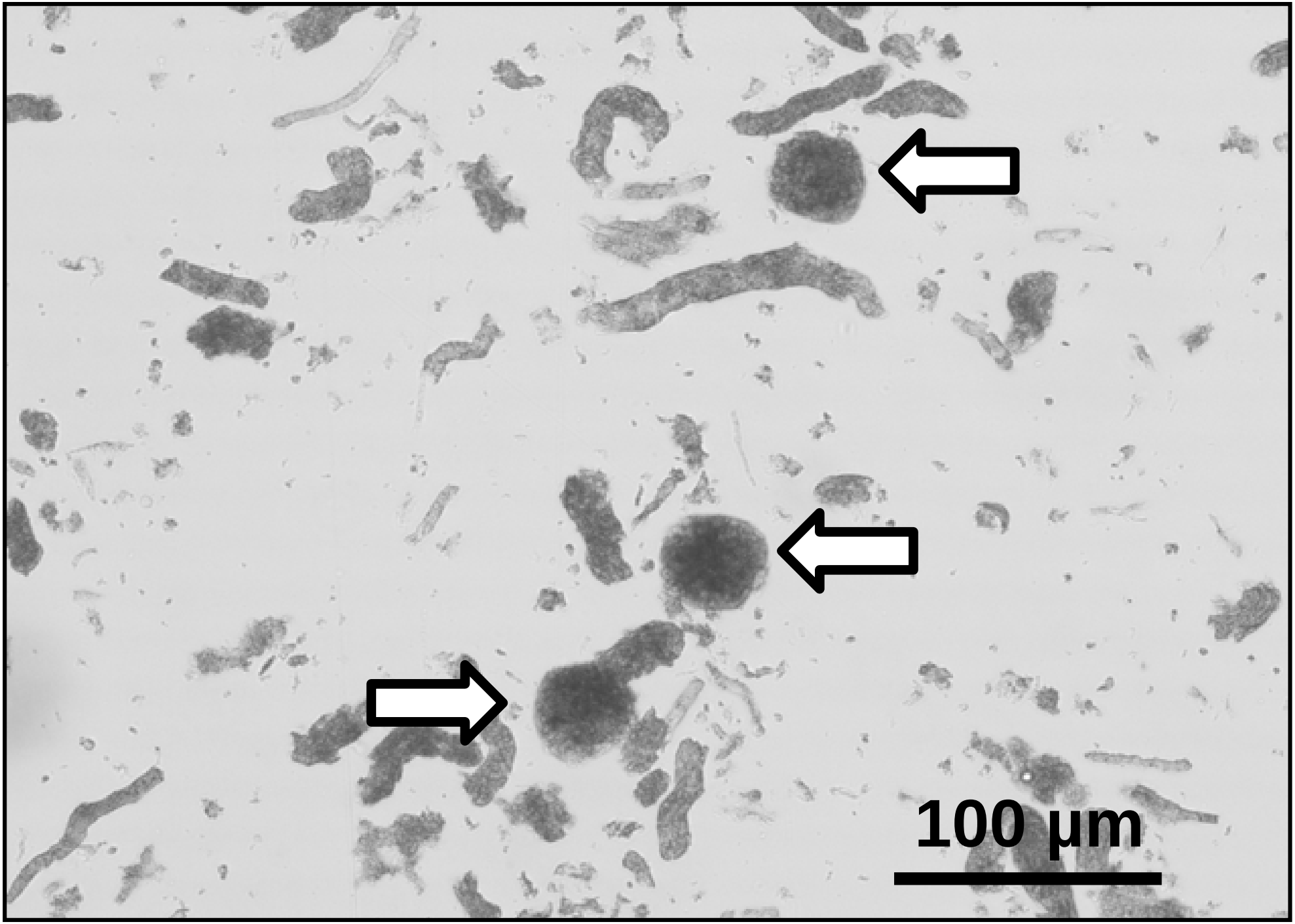
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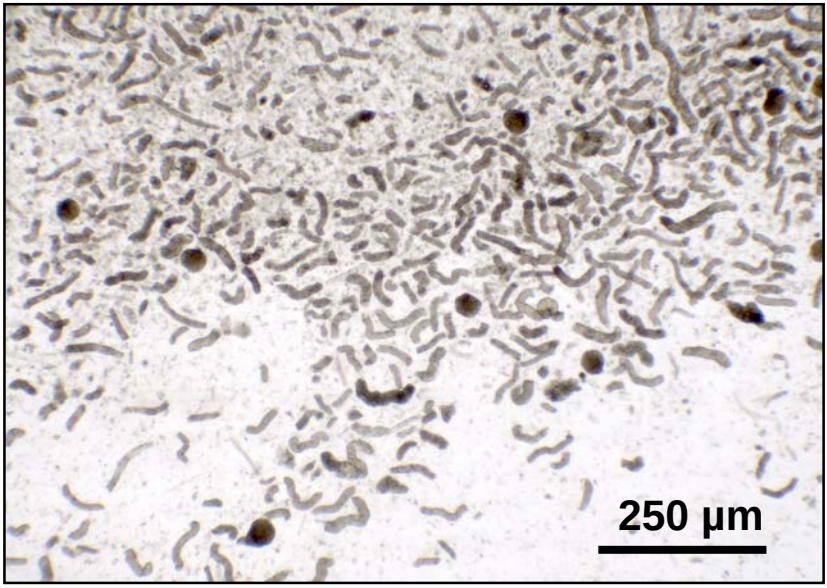
A.



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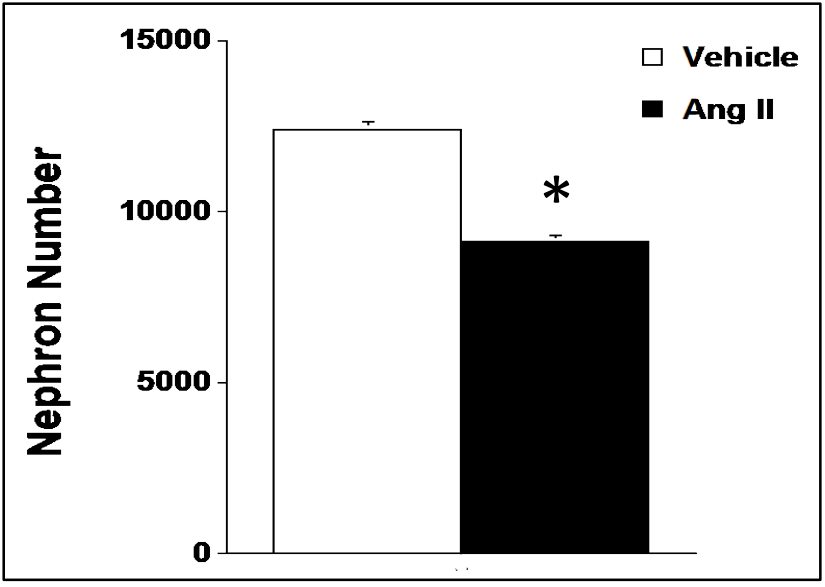

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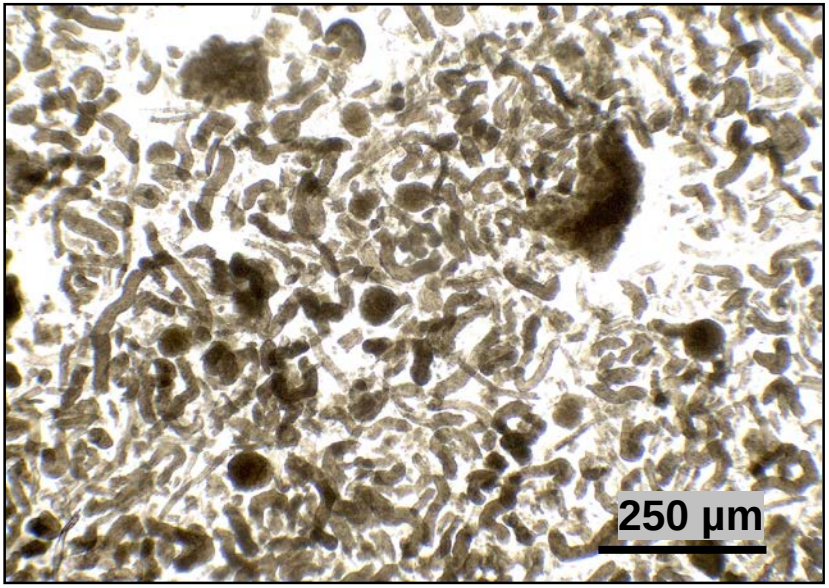

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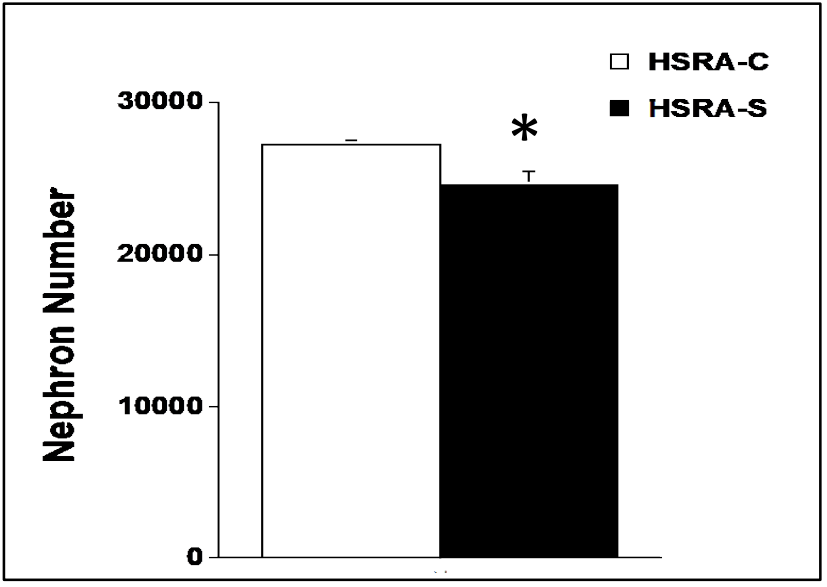

Table 1

Click here to download Table Didion_JoVE_Table_080618.xlsx

Species	Nephron Number per Kidney	Reference
Mouse	9,000-21,000	18-22,28
Rat	13,000-27,000	31,33
Sheep	200,000-800,000	23,29,34
Pig	1,600,000-4,600,000	32,35
Human	500,000-2,000,000	8-15

Table of Materials

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Name	Company
Isoflurane anesthesia	Abbott Laboratories
Isoflurane vaporizor system & flow gauge	Braintree Scientific
Leica Inverted Microscope DMIL LED	Leica Microsystems
Digital water bath	Fisher Scientific
ToughCut Fine surgical scissors	Fine Science Tools
Micro dissecting forceps 4 1/4 in.	Biomed Res Instruments, Inc
Plexiglass board 5 in. x 7 in.	any source suitable
Hexagonal polystyrene weighing dish	Fisher Scientific
Razor blades	Fisher Scientific
Gauze sponges 4 x 4 in. 8 ply	Fisher Scientific
10x concentrate phosphate buffered saline (PBS)	Sigma Aldrich
6 N Hydrocholric acid solution	Sigma Aldrich
15 mL conical centrifuge tube	Fisher Scientific
50 mL conical centrifuge tube	Fisher Scientific
Disposable 5 mL syringe	Cole Palmer
18G1.5 disposable needle	Fisher Scientific
21G1.5 disposable needle	Fisher Scientific
12-well multiple-well cell culture plates with lid	Cole Palmer
Polypropylene modular test tube rack	Cole Palmer

Catalog Number	Comments
05260-05	
VP I	Include medical grade oxygen supply
DMIL LED	Any make also suitable
2239	Any make also suitable
14058-11	25 mm cutting edge, 11.5 cm length; Tips: sharp-sharp; Tip shape: stra
10-1760	Curved tip
n/a	Any make also suitable
02-2002-100	Any make also suitable
12-640	Single edge carbon steel 0.009
MSD-1400250	
P5493-4L	Dilute to 1x
3750-32	
14-959-70C	Any make also suitable
14-959-49A	Any make also suitable
EW-07944-06	Any make also suitable
14-826-5D	Any make also suitable
14-826-5B	Any make also suitable
#FW-01959-06	Any make also suitable
#EW-06733-00	Capable of accommodating 15 and 50 mL conical tubes; any make also

ıight

› suitable

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Response to Editorial and Reviewer Comments

We would like to thank the Editorial team and the reviewers for their helpful comments and suggestions. Below are the detailed responses to their suggestions and comments.

The following Editorial Comments have been addressed:

1. The manuscript has been carefully edited to limit both spelling and grammatical errors.
2. Panels A and B in Figures 2 and 3 panels have been combined.
3. Error bars in Figures 2 and 3 have been defined as the standard error of the mean (SE).
4. City, State, Zip Code and Country are now all included in the institutional address.
5. Email addresses have been included for all authors:
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   Xuexiang Wang; email: xuexiang_Wang@rush.edu
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   Sean P. Didion; email: sdidion@umc.edu
6. Where appropriate abbreviations have been standardized per the Journal.
7. Spaces have been included between numbers and corresponding units.
8. Protocols have all been labeled numerically per Journal requirements.
9. All pronouns have been removed from text.
10. Solutions, materials, and equipment information have been moved to Materials Table in excel format.
11. Information regarding age, gender, and strain of mice has now been included.
12. All surgical instruments have been specified.
13. Lines 218-220 of text have now been included as a note.
14. Critical steps in protocol have been discussed in the Discussion section.
15. Journal titles in Reference section have all been spelled out completely.
16. Table of supplies have now been expanded to include source and company information.

Reviewer #1

1. The reviewer noted no major concerns but suggested including some additional discussion of limitations associated with the acid maceration method and correction of grammatical errors.

We thank the review for their suggestion regarding additional limitations, particularly in regarding to acid digestion of glomeruli that may be more sensitive to

digestion by acid due to disease. We have included some additional text to this in the Discussion section. In addition, we have worked to correct all grammatical errors.

Reviewer #2

1. The reviewer noted that the acid maceration is documented approach to estimate nephron number and to clarify any technical modifications that are different or new to established protocols.

As we acknowledged in our paper the acid maceration method presents is well established and we made small modifications to that by others. The main goal of this manuscript and subsequent demonstration is to present the method for others in a visual manner. The authors were invited by the Editors to present the method as a way to make the method and materials required more accessible to the scientific community.

2. The reviewer suggests correcting the grammatical errors contained within the manuscript.

Per the reviewer's suggestion we have worked to correct all grammatical errors in the revised manuscript.