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Micro-computed tomographic analysis of the radial geometry of intrarenal artery-vein pairs in rats and rabbits: comparison with light microscopy

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Abstract

We assessed the utility of synchrotron-radiation micro-computed tomography (micro-CT) for quantification of the radial geometry of the renal cortical vasculature. The kidneys of nine rats and six rabbits were perfusion fixed and the renal circulation filled with Microfil®. In order to assess shrinkage of Microfil®, rat kidneys were imaged at the Australian Synchrotron immediately upon tissue preparation and then post fixed in paraformaldehyde and reimaged 24 hours later. The Microfil® shrank only 2-5% over the 24 hour period. All subsequent micro-CT imaging was completed within 24 hours of sample preparation. After micro-CT imaging, the kidneys were processed for histological analysis. In both rat and rabbit kidneys, vascular structures identified in histological sections could be identified in two-dimensional (2D) micro-CT images from the original kidney. Vascular morphology was similar in the two sets of images. Radial geometry quantified by manual analysis of 2D images from micro-CT was consistent with corresponding data generated by light microscopy. However, due to limited spatial resolution when imaging a whole organ using contrast-enhanced micro-CT, only arteries ≥100 μm and ≥60 μm in diameter, for the rat and rabbit respectively, could be assessed. We conclude that it is feasible and valid to use micro-CT to quantify vascular geometry of the renal cortical circulation in both the rat and rabbit. However, a combination of light microscopic and micro-CT approaches are required to evaluate the spatial relationships between intrarenal arteries and veins over an extensive range of vessel size.

Keywords: counter-current exchange, renal circulation, synchrotron radiation

Introduction

Synchrotron-radiation micro-computed tomography (micro-CT) can be used to generate three-dimensional (3D) reconstructions of tissue vasculature. Realistic 3D geometric information is vital for development of accurate models of blood and solute transport throughout the vasculature and into the surrounding tissue. In theory, high-resolution two-dimensional (2D) histological images generated by light microscopy can be aligned and a 3D geometry reconstructed. However in practice, the alignment, and histological artefacts usually makes the reconstruction difficult. Confocal microscopy offers another option that overcomes these shortcomings, but is generally limited in its depth of field to about 150 μm, and by the number of light channels for imaging tissue fluorophores. For these reasons, micro-CT is often the most efficient approach for 3D reconstructions of the vasculature, particularly for regional or organ-scale reconstructions.

To obtain a micro-CT image, a radio-opaque contrast agent such as the polymer Microfil® is infused into the vasculature. One of the practical limitations of this approach is the potential for Microfil® to segregate and to shrink.[1-3] Segregation may occur if the Microfil® components are not completely mixed, while shrinkage is particularly a problem if the tissue processing protocol involves exposure to solvents such as ethanol.[3] Thus, questions arise over the potential for volume changes in the Microfil® between the time of infusion, its curing, and

sample imaging. Volume changes will compromise the ability to interpret the processed micro-CT image, particularly in the radial dimension (lumen) of vessels. This becomes even more problematic when structural data obtained from the micro-CT image are used for transport analysis. Small changes in the distances between vascular and extravascular elements can have disproportionately large effects on estimated transport rates.

One such case of transport analysis is the diffusive arterial-to-venous (AV) shunting of oxygen in the renal vasculature. Throughout the mammalian kidney, veins are often seen to partially wrap a nearby artery (geometric wrapping).[4, 5] This structural arrangement could potentially enable AV shunting of various gases.[5] The amount of shunting that can occur is extremely sensitive to the distance between each artery-vein pair[4, 6] and to the degree of geometric wrapping.[4] Furthermore, estimation of the rate of blood flow, and thus the time available for shunting to occur,[4, 7] is also strongly dependent on accurate measurement of the cross-sectional areas of vessels. Indeed, uncertainty in the data regarding the 3D structure of the vasculature has contributed significantly to our current uncertainty as to the magnitude of renal AV oxygen shunting.[8, 9]

There is experimental evidence that diffusive AV oxygen shunting in the renal vasculature may be significant.[10-12] However, the quantitative significance of this phenomenon remains a matter of controversy.[5, 8, 9, 13, 14] Currently, it is not possible to accurately measure AV oxygen shunting experimentally. For this reason, we rely on mathematical modelling to investigate this phenomenon by simulating diffusion and convection of oxygen within the renal cortex.

All available models of oxygen transport in the renal cortex[4, 7, 13-16] are based on the eleven branching orders, termed 'Strahler orders', of the rat renal circulation defined by Nordsletten *et al.*[17] using the micro-CT data of Garcia-Sanz and colleagues.[18] Shrinkage of Microfil® likely confounded the observations of Garcia-Sanz and colleagues due to tissue immersion in alcohols.[18] The consequence of shrinkage of Microfil® in the study by Garcia-Sanz *et al.* is that the diameters of arteries and veins presented in the work of Nordsletten *et al.*[17] are underestimates of true vascular dimensions, thus limiting the accuracy of a number of computational models of renal oxygenation.[7, 13] To avoid this limitation, we recently quantified the degree of shrinkage of Microfil® that would be expected from the protocols followed by Garcia-Sanz *et al.* (~22%) and used these to correct vascular dimensions in a pseudo-three dimensional model of oxygen transport in the kidney of the rat.[14, 15] However, development of computational models that can be applied to other species will ideally require methods for quantifying vascular geometry that avoid shrinkage artefacts. Therefore, in the current study we sought to compare geometric data regarding the renal vasculature generated from micro-CT imaging with those generated by 2D light microscopy. Both qualitative and quantitative comparisons were made using the same tissue samples from both rats and rabbits. We explored techniques for utilising Microfil® to understand and avoid shrinkage. We also determined whether, in the 24 hours after preparation of the tissue, there is appreciable shrinkage and/or deterioration of vascular casts generated with either cured or uncured Microfil®. In addition, by

determining the accuracy with which 2D micro-CT images correlate with 2D histological images, we were able to distinguish true vessel boundaries visualised in micro-CT images. The inclusion of studies in rabbits provides the first step towards development of models of renal oxygen transport in species other than rats, with a view to eventually developing a model applicable to humans.

Results

Quality of micro-CT reconstructions and effects of excluding the curing agent

Using rat kidneys, we first determined whether Microfil® can be used without addition of the curing agent, thus avoiding the effects of shrinkage. We also determined whether, in the 24 hours after preparation of the tissue, there is appreciable shrinkage and/or deterioration of vascular casts generated with either cured or uncured Microfil®. Nine rat kidneys were perfusion fixed and the renal vasculature infused with Microfil®. The curing agent allows for the polymerisation of Microfil® into a silicone compound, and once polymerised the silicone can undergo shrinkage. Thus, kidneys were either infused with Microfil® compound that included (n=6) or excluded (n=3) the curing agent. The quality of visualisation of the renal circulation was greatly affected by the addition of curing agent to the Microfil® compound (Fig. 1). The vasculature of the rat kidneys containing Microfil® with curing agent could be clearly visualised in the scans taken both immediately after (Fig. 1G, I, K) and 24 hours after (Fig. 1H, J, L) sample preparation. The quality of visualisation was sufficient to resolve vessels with a diameter ≥100 μm. There was very little evidence of changes in the morphology of the Microfil® across the 24-hour period. In contrast, while the three rat kidneys prepared without curing agent had reasonable, albeit somewhat patchy, visualisation in the immediate scan (Fig. 1A, C, E), visualisation was poor in the scans 24 hours after sample preparation (Fig. 1B, D, F). Consequently, subsequent analyses of the rat kidneys only included the 6 kidneys containing Microfil® and its curing agent.

Because vascular morphology was preserved in the rat kidneys for 24 hours only when curing agent was included, curing agent was included in the Microfil® used in subsequent studies in rabbits. The quality of visualisation of the renal circulation of the rabbit kidney was sufficient to resolve vessels with a diameter ≥60 μm (Fig. 2).

Shrinkage of Microfil®

In rat kidneys, a quantitative analysis of the shrinkage of Microfil® demonstrated a small but statistically significant degree of shrinkage over the 24 hours between the two scans (Fig. 3). Our first analysis involved measuring the widths of vessel pairs. That is, the width of an artery, vein and the space between the two vessels. Using Drishti, a volume rendering and analysis software, the widths of a total of 244 vessel pairs in the six micro-CT reconstructions were compared. The overall mean width 24 hours after sample preparation (594 ± 16 μm) was 3.3 ± 0.6% less than that determined immediately after sample preparation (621 ± 18 μm; P <0.001 by Student's paired t-test; Fig. 3A). In our second analysis, we measured the diameters of

only arteries in a smaller subset of the 3D volumes. When only arterial diameter was measured, it was found to reduce by 2.2 ± 0.4%, from 377 ± 13 μm to 368 ± 13 μm over the 24-hour period (P <0.001; Fig. 3B).

In order to better characterise the degree of shrinkage across the 24 hours after sample preparation, ordinary least products regression analysis was performed.[19] Theoretically an artery-vein pair of zero width should maintain zero width after 24 hours, assuming no physical matter is added to it. Thus, the relationship between paired vessel diameter at the two time-points should theoretically pass through the origin. We therefore used the ordinary least products method to generate a line in the form of $y = bx$, where y = the diameter of vessels 24 hours after sample preparation, x = diameter of vessels from the immediate scan, and b = the slope of the relationship between x and y. For the analysis of the widths of vessel pairs, the line of best fit was $y = 0.95x$ (Fig. 3C), with 95% confidence limits of the slope that excluded unity (95% CI: 0.94 – 0.96). Thus, this regression analysis suggests that Microfil® shrinks ~5% following 24 hours of tissue preparation. In the analysis of changes in arterial diameter across the 24 hours after sample preparation, ordinary least products regression (Fig. 3D) provided a line of best fit with the equation $y = 0.98x$. The 95% confidence intervals of the slope excluded unity (0.97 – 0.99). Hence, this analysis suggests that Microfil® shrinks by ~2% during the 24 hours after tissue preparation.

Qualitative comparison of vessel geometry using micro-computed tomography and light microscopy

In both rat and rabbit kidneys, vessels identified in the histological sections could also be identified in corresponding images obtained by micro-CT (Figs. 4 and 5). Glomeruli seen in the histological sections of the rat kidney (Figs 4C, E, G, M, O, Q, S), were for the most part also observed in the micro-CT images as bright white dots (Figs 4D, F, H, N, R). In the light micrographs, Microfil® could be identified in most arteries and veins, as well as capillaries. In the rat micro-CT images, rings were observed at the edge of the vessels due to a phase-contrast effect. These rings are herein referred to as 'edge effects'. The edge effects are part of the true diameter of the vessels (as determined by the quantitative analysis described later), and are present in these images because the samples were placed at a relatively long distance from the detector (see Methods section for details).

Quantitative comparison of vessel geometry in rat kidneys using micro-computed tomography and light microscopy

Measurements of arterial diameter, diffusion distance (the shortest distance from the lumen of the artery to its adjacent vein), and the proportion of the wall of the artery in contact with the wall of a paired vein (geometric wrapping) determined from micro-CT images correlated strongly with corresponding measurements determined by light microscopy (Fig. 6). For measurements of arterial diameter, diffusion distance, and geometric wrapping, two approaches were used. The first involved including the edge effects in all measurements (i.e. the outer edges of the edge effect were included in the measurements). The second approach

involved excluding the edge effect from the measurements. That is, the inner edges of the edge effect were the limits for the measurements. Because the relationship between measurements generated using the two approaches might not necessarily pass through the origin, the ordinary least products method was used to generate a line of the form $y = bx + c$, where y = the variable measured in the micro-CT images, x = the variable measured in the histological images, b = the slope, and c = the intercept at $x = 0$.

For the analysis of arterial diameter including the edge effect (Fig. 6A) the line of best fit was $y = 1.10x + 14.35$. The 95% confidence limits of the slope included unity (95% CI: 0.93 – 1.27) and the 95% confidence limits of the y-intercept included zero (95% CI: -37.52 – 66.21). For the analysis of arterial diameter excluding the edge effect observed in the micro-CT images, the line of best fit was $y = 0.96x – 55.77$. The 95% confidence limits of the slope included unity (95% CI: 0.81 – 1.11) but the 95% confidence limits of the y-intercept excluded zero (95% CI: -101.56 to -9.98). Thus, we could detect fixed bias in the micro-CT method compared with light microscopy only if the edge effect was excluded. Importantly, no proportional bias could be detected, regardless of whether the edge effect was included in the analysis. Nevertheless, arterial diameter was on average 11.2 ± 4.9 μm less when the edge effect was excluded than when it was included (P <0.001, paired t-test).

On average, diffusion distance was 45.6 ± 3.0 μm less when measurements from micro-CT images included the edge effect than when it was excluded (P <0.001, paired t-test). When the edge effect was included, average diffusion distance measured from micro-CT images did not differ significantly from that measured by light microscopy (P = 0.89, paired t-test). Nevertheless, both fixed (y-intercept) and proportional (gradient) bias were detected by ordinary least products regressions analysis (Fig. 6B). For the analysis of diffusion distance when the edge effect was included, the line of best fit was described by the equation $y = 0.76x + 5.71$. The 95% confidence intervals of the slope excluded unity (95% CI: 0.53 – 0.98), and those of the y-intercept excluded zero (95% CI: 0.20 – 11.22). Thus, measurement of diffusion distance from micro-CT images may generate an overestimate when diffusion distance is small, but may underestimate larger diffusion distances (Fig. 6B). For the analysis of diffusion distance excluding the edge effect, the line of best fit was $y = 2.11x + 20.62$. The 95% confidence limits of the slope excluded unity (95% CI: 1.13 – 3.09), although the 95% confidence limits of the y-intercept included zero (95% CI: -3.11 – 44.34). Thus, we detected proportional but not fixed bias, indicating an overestimation of diffusion distance when the edge effect was excluded in our measurements.

In the analysis of geometric wrapping, including and excluding the edge effect, wrapping measured by the manual micro-CT method did not differ significantly from that measured by the light microscopic method (P = 0.18 and P = 0.09 respectively, paired t-test). When including the edge effect, ordinary least products regression (Fig. 6C) provided a line of best fit with the equation $y = 1.08x – 4.13$. The 95% confidence intervals of the slope included unity (0.80 – 1.37) and the 95% confidence limits of the y-axis included zero (95% CI: -14.22 – 5.97). Analysis of wrapping excluding the edge effect provided a line of best fit described by $y = 1.01x – 2.65$.

Again, the 95% confidence intervals of the slope included unity (0.68 – 1.35) and the 95% confidence intervals of the *y*-intercept included zero (-14.51 – 9.21). Thus, we were unable to detect significant fixed or proportional bias in measurements of wrapping obtained from micro-CT images, regardless of whether the edge effect was included or excluded.

Discussion

We have shown that measurements of radial vascular geometry derived from micro-CT images of kidneys containing intravascular Microfil® correspond closely with those derived from histological images. In previous studies in which micro-CT has been used to quantify renal vascular geometry, kidney tissue was processed in a way that would have resulted in shrinkage of the polymer.[17, 18] In these studies, such an approach precludes the generation of meaningful data regarding the radial geometry of artery-vein pairs. In contrast, our current approach has the potential to allow accurate characterisation of both radial and axial geometry of the cortical vasculature in multiple species. We also found negligible shrinkage of Microfil® across a 24-hour period after tissue preparation for micro-CT imaging. Thus, micro-CT images can be used for quantitative analysis of vascular geometry up to at least 24 hours after tissue preparation.

By omitting the curing agent in three of the rat kidneys, we aimed to generate a 3D reconstruction of the renal cortical vasculature free of the effects of shrinkage or breakage of the polymer. However, it was apparent that the kidneys that excluded curing agent were not adequate for analysis following 24 hours. Many vessels were no longer visualised 24 hours after infusion of the Microfil®. There were regions of vessel sparseness, suggesting that the Microfil® solution had separated into two components: the MV-Compound and the MV-Diluent. These compounds are mixed before infusion. However, it is highly likely that, in the absence of curing agent, the heavier component (MV-Compound), which contains lead chromate and can thus be visualised by synchrotron radiation, sinks to the bottom, while the MV-Diluent rises to the top. On the basis of these findings, we recommend that cured Microfil® should always be used in preference to non-cured Microfil®, unless the micro-CT scans are conducted immediately upon tissue preparation. Furthermore, as a consequence of these preliminary observations, subsequent analyses focused only on those kidneys that were filled with Microfil® containing curing agent.

An important consequence of the requirement for curing agent in studies using Microfil® is that we cannot account for shrinkage that might occur in our samples during the curing process. However, this effect is likely small, since we demonstrated close agreement between measures of vascular geometry obtained from micro-CT images and those obtained from light microscopy. Furthermore, shrinkage of vessel diameter of only 2-5% was observed over the 24 hours following tissue preparation.

A major limitation of previous studies in which renal vascular geometry was quantified by micro-CT[17] was that the tissues were exposed to organic solvents which would promote shrinkage of Microfil®. Indeed, by using the same solutions as that used by Garcia Sanz and colleagues we have estimated that the processing of the tissues analysed by Nordsletten and colleagues would have led to ~22% shrinkage.[14] Therefore, in the current study we avoided use of such solvents. Using multiple analytical approaches, we found only 2-5% shrinkage of Microfil® in the 24 hours after preparation of the tissue for micro-CT. We consider this shrinkage negligible, but even if it were considered significant, by documenting it we generate the possibility that it can be accounted for if necessary. Access to micro-CT facilities, particularly those that rely on synchrotron radiation, can be restrictive. Thus, it is often necessary to prepare material before the scanner becomes available. Our current findings provide confidence that valid geometric data can be obtained from renal tissue prepared with Microfil® up to 24 hours before scanning. Unfortunately, a limitation is that we do not know how much shrinkage occurs beyond 24 hours, so our findings cannot be applied to micro-CT scans completed more than 24 hours after tissue preparation.

One challenge we faced in the analysis of these data was the presence of an edge effect in some of the micro-CT images generated from the kidneys of rats. These arose because the specimens were placed 3.24 m from the detector in those particular studies. In later studies of both rat and rabbit kidneys the specimens were placed much closer to the detector (0.38 m) so no edge effect was observed. Nevertheless, our analysis provides strong evidence that the outer boundary of the edge effect corresponds to the outer edge of the blood vessel. We found that when the edge effect was excluded from our analysis of the micro-CT images, arterial diameter was underestimated, and diffusion distance overestimated. Our analysis when the edge effect was included suggests that measurements of diffusion distance from micro-CT images may be overestimated when diffusion distance is small, but may be underestimated for larger diffusion distances. This may be because images of artery-vein pairs could not be matched perfectly. Despite this, when the edge effect was included there was very close agreement, between measurements generated by micro-CT compared with light microscopy, for both arterial diameter and diffusion distance. Interestingly, we could not detect a significant difference in proportion of wrapping dependent on whether the edge effect was included or not. This results from the fact that the width of the edge effect is consistent across both arteries and veins, where the mean width of the edge effect was 44.1 ± 7.6 μm and 44.2 ± 7.3 μm in the artery and corresponding vein respectively ($P > 0.05$).

Our current findings indicate that the morphology of the vasculature in rats and rabbits, determined from micro-CT images, closely resembles vessel geometry determined from light microscopy. To quantitatively assess the validity of the micro-CT approach, we determined the relationships between spatial characteristics determined by micro-CT and light microscopy. There was a strong correlation between the two methods, demonstrating that micro-CT is a valid and accurate method for quantifying the spatial characteristics of the renal circulation. Indeed, no fixed or proportional bias, for micro-CT relative to light microscopy, was detected for measurements of arterial diameter or proportion of wrapping. However, we did detect proportional bias for diffusion distance. Nevertheless, the mean estimate of diffusion distance

did not differ significantly between the two methods (22.8 ± 1.3 μm vs. 22.6 ± 1.7 μm from the micro-CT and histological images respectively), and the level of bias was small.

Micro-CT appears to be particularly useful for analysis of larger vessels, since it is possible to follow the course of these vessels in 3D reconstructions, overcoming the limitation imposed on light microscopy by the fact that there are few large vessels in a single 2D section of the kidney.[4] On the other hand, the high resolution from the light microscopic images is able to provide details about the parenchyma as well as visualisation of the structure of the vessel wall, so we could determine whether the vessels in the micro-CT images were arteries or veins. Furthermore, light microscopy allows for visualisation of the smaller vessels. However, the 2D nature of information generated by light microscopy represents a significant limitation. A major advantage of micro-CT is the potential to follow the course of the circulation, and thus define both axial and radial geometry of vascular systems. Thus, micro-CT and light microscopy appear to be complementary techniques. By combining these approaches, information on the axial and radial geometry of both large and small vessel-pairs in the renal circulation can be generated.

We confirmed that Microfil® perfused the smallest microvessels in both species by allowing the Microfil® to flow freely out of the renal vein. However, in the case of the rat experiments, the low concentration of radiopaque lead chromate in the lumen of the microvessels, in addition to the relatively higher background X-ray absorption of the interstitial tissue, reduced our ability to resolve vessels ≤100 μm in diameter without ambiguity. In contrast, small cortical radial arteries were observed extending towards the surface of the kidney in the rabbit, which enabled resolution of vessels ≥60 μm in diameter. In addition, several factors tend to confound the acquisition of micro-CT data sets across the entire renal vasculature, even in smaller samples from rodents. These confounding factors are especially important at the high resolution (submicron to several micrometres) required for assessment of vascular geometry and the spatial organisation of arteries and veins. First, the highest resolution achievable for an intact kidney is set by the pixel array size of the detector in the horizontal direction (generally 1,024 pixels), since synchrotron radiation is widest in the horizontal direction (orders of cm horizontally and orders of mm vertically). Therefore, whole rat kidneys are acquired at 10-12 μm pixel resolution. Second, X-ray absorption of the larger volume of Microfil® that fills large renal arteries is many times greater than that of the small volumes in renal arterioles, preventing adequate contrast of the smallest arteries when large arteries are present in the same field of view. Such image saturation can only be overcome by collecting CT images of small biopsies of fixed tissue to visualise the microvessels. Hence, the best prospects to non-destructively assess renal vessel organisation over the largest range of vessel sizes in all regions of the kidney is limited to ~10 μm pixel resolution with conventional X-ray detectors.

In the current study, synchrotron-radiation micro-CT provided suitable imaging of vessels with a diameter ≥100 μm and ≥60 μm in whole rat and rabbit kidneys respectively. An alternative imaging approach may be required to resolve smaller vessels. Low energy imaging is better suited for imaging samples to a submicron level. Thus, using dedicated micro-CT

beamlines at facilities such as the Swiss Light Source may be a better option when imaging smaller vascular structures, as submicron resolution imaging has been successfully achieved.[20] Also, nano-computed tomography has been used to successfully achieve a resolution of 0.5 μm in the mouse kidney.[21] Nevertheless, both light microscopy and micro-CT approaches are required to assess the spatial relationships over a wide range of vessel diameters. These tools provide the basis for detailed characterisation of both axial and radial geometry of artery-vein pairs that can in turn inform sophisticated 3D or pseudo-3D models of oxygen transport in the renal cortex. However, manual generation of these measurements is very labour-intensive. Thus, there is now a need for development of an algorithm for automated analysis of vascular geometry from data-sets generated by micro-CT. Such a method would facilitate rapid development of sophisticated models of oxygen transport in the renal cortex in multiple mammalian and even non-mammalian species, and in a range of disease states.

In conclusion, our current findings suggest it is feasible and valid to use micro-CT to gather information on the spatial relationships between arteries, veins and the renal parenchyma in the kidneys of rats and rabbits. Computational models are only as useful and as good as the data they are based on. Some existing models therefore should be re-analysed with accurate data forming the basis of the model. Our findings provide a method that can be used for quantification of vascular geometry in multiple species, and thus development of species-specific models of renal oxygen transport.

Methods

Overview

To assess the effects of the curing process on shrinkage, rat kidneys were perfusion fixed and filled with Microfil®. A component of the shrinkage of Microfil® is known to be due to the curing process.[22-24] Thus, rat kidneys were perfusion fixed and filled with Microfil® that either included (n = 6) or excluded (n = 3) the curing agent, and then scanned to produce micro-CT images at two time points; immediately upon tissue preparation, and 24 hours later. Diameters of vessels were measured manually at both time points to determine the degree of shrinkage.

To determine whether the synchrotron micro-CT approach is valid, rabbit kidneys (n = 6) were also prepared in an analogous manner to the rat kidneys. A comparative analysis was then performed between 2D histological kidney sections (examined by light microscopy) and 2D micro-CT sections from the micro-CT scans. To do this, following the micro-CT scans of both rat and rabbit kidneys, 3 μm sections were cut from four regions of each kidney, stained with haematoxylin and eosin, imaged by Scan Scope (Aperio, Vista, CA, USA) and viewed by a virtual microscope (ImageScope, version 11.2.0.780, Aperio Technologies).

Animals

Nine male Sprague Dawley rats (weighing 250 - 300 g) and five adult male and one adult female New Zealand White rabbits (weighing between 2.5 - 3.2 kg) were used in this study. All procedures involving animals were approved by the Animal Ethics Committee of the Monash University Animal Research Platform and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Rat kidneys

Tissue preparation: We used methods similar to those employed in our previous study.[4] Nine male Sprague Dawley rats were anaesthetised with sodium pentobarbitone (60 mg/kg. i.p; Sigma-Aldrich, St Louis, MO, USA). A cannula was inserted retrogradely into the abdominal aorta and the kidneys were then cleared with phosphate buffer and perfusion fixed at physiological pressure with Karnovsky's fixative (4% paraformaldehyde and 4% glutaraldehyde in 0.2 M phosphate buffer). Microfil® (10-12 ml; MV-122; Flow Tech, Carver, MA, USA) was then infused into the kidney until the kidneys were uniform in colour and the Microfil® flowed freely from the veins. The renal artery and vein were then ligated. The kidneys were removed, decapsulated, and weighed, before being immersed in fixative, and transported immediately to the Imaging and Medical Therapy Beam Line (IMBL) at the Australian Synchrotron.

Micro-CT scanning and imaging: The CT scans were conducted in enclosure 3B at IMBL. Using the 'Ruby' detector, the X-ray photon energy was tuned to 32 keV. The detector is a photo-sensitive device coupled by a bright lens to a suitable X-ray sensitive scintillator. The system was conceived by the IMBL team and designed and fabricated at Monash University in the Division of Biological Engineering at the Laboratory for Dynamic Imaging (LDI). A PCO.edge sensor is mounted on a vertical motor-driven slide set within a light-tight enclosure. A mirror is used to view a phosphor plate set orthogonally to the direction of the beam. This allows protection of the sensor from direct and scattered beam radiation using suitable high-Z materials. For this experiment the sensor was equipped with a Nikon Micro-Nikkor 105 mm/f 2.8 macro lens allowing the slide to be used as a zoom control. The scintillator was a 12 mm-thick terbium-doped gadolinium oxy-sulfide (Gadox, P43) screen with aluminium powder coat as an optical block. During the experiment the system was tuned to produce 2560 × 2160 pixel images. This resulted in a field of view of 15 × 12 mm with 6.1 μm pixel size and a measured resolution of 20.1 μm. CT data acquisition for the samples consisted of two scans, each containing 1800 projections over the arc of 180 degrees. Multiple scans were needed to accommodate the full image of the sample, which was larger than the size of the vertical beam size. The images from the scans acquired at the same rotation but different vertical position were stitched in the post-processing step to form the full image. Under these conditions the exposures were 300 ms per projection, and the accumulated time taken to scan a single sample was approximately 45 minutes. Each scan included 20 images of background (no sample in the beam) and 20 images of dark-field (beam is off) contrasts for further processing.

For the rat kidneys, there were two rounds of 'beam-time' in our study. In the first round of beam-time, we aimed to determine whether phase-contrast could enhance the edges of the vessel. To do this, whole kidney samples (n = 6; with curing agent = 3, without curing agent = 3) were placed 3.24 m away from the detector as the distance between a sample and the detector affects images of the edges of the sample. This resulted in the generation of an 'edge effect' (see Results). Therefore, during the second round of beam-time, whole kidneys (n = 3, all with curing agent) were placed much closer (0.38 m) to the detector, to avoid the edge effect. To determine the amount of shrinkage of the Microfil® within a 24-hour period, two scans were completed for each kidney: one immediately after tissue preparation and one 24 hours later.

Rabbit kidneys

Rabbits are commonly used as an experimental model in renal oxygen transport research, and consequently there is an abundance of experimental data based on this species.[25-28] Thus, quantification of vascular geometry in the kidney of the rabbit can provide the basis for development of mathematical models to allow deeper interrogation of the physiological significance of these experimental findings. Furthermore, unlike the kidneys of dogs or sheep, the kidneys of the New Zealand white rabbit are relatively small (approximately 35 mm in length).[29] This is advantageous for micro-computed tomographic imaging, as using a smaller sample allows us to scan the whole kidney at a relatively higher resolution.

Tissue preparation: Lignocaine (1% w/v; Xylocaine; AstraZeneca; North Ryde, NSW, Australia) was injected intradermally around the marginal ear vein and central ear artery of the left ear. A 20- and a 22-gauge catheter (Surflo® Intravenous Catheter, Terumo Corporation; Tokyo, Japan) were inserted transcutaneously into the artery and vein, respectively. The rabbits were then anaesthetised with sodium pentobarbitone (15 mg/ml solution, 90-150 mg plus 30-50 mg/h i.v.; Sigma-Aldrich, Sydney, Australia) and intubated (Portex® Cole Neonatal Tube, ID = 2.5 mm, OD = 4.0 mm; NSW, Australia) to permit artificial ventilation (Harvard Apparatus Model 683; South Natick, MA, USA) with a tidal volume of 15 ml and a ventilation rate of 33-60 breaths/min. The arterial line was then connected to a pressure transducer. Level of anaesthesia was assessed using the corneal reflex and jaw tone test. Oxygen saturation of arterial haemoglobin (SO_2) was assessed by pulse oximetry (Model 8600V, Nonin; Plymouth, MN, USA). If required, ventilation rate and depth of anaesthesia were adjusted to maintain SO_2 ≥98% and mean arterial pressure at 60-90 mmHg.

The left kidney, abdominal aorta, and inferior vena cava (IVC) were exposed by a flank incision and heparin (10,000 IU, Pfizer Pty Ltd, Bentley, Australia) was administered intravenously. A large bore catheter (PVC, ID = 2.00 mm, OD = 3.00 mm; Critchley Electrical Products, NSW, Australia) was placed retrogradely in the abdominal aorta, and the kidneys were perfused with ~200 ml of phosphate buffer at 100 mmHg. The perfusate was then switched to 4% w/v paraformaldehyde to perfusion fix the kidneys. The kidneys were then perfused with ~30 ml of Microfil® (MV-122; Flow Tech, Carver, MA, USA) until they had a uniform colour and the Microfil® flowed freely from the veins. The renal arteries and veins

were then ligated, and both kidneys were removed, decapsulated, and weighed. The rabbit kidney was immersed in fixative and scanned at the IMBL at the Australian Synchrotron within 24 hours of tissue preparation.

Micro-CT scanning and imaging: Rabbit kidneys were imaged at the IMBL at an energy level of 30 keV. The experimental setup and parameters were the same to those described above for the rat kidneys except the pixel size was 15.6 μm, providing a resolution of 32.7 μm.

Three-dimensional volume reconstruction and rendering of whole rat and rabbit kidneys

Prior to rendering the whole kidney in 3D, each of the 5000+ image slices were first visually inspected in ImageJ (http://imagej.nih.gov/ij/). The post-processing of the data included several steps. The first step involved forming clean background and dark-field images as the median of all 20 repeats. Then they were applied to all raw sample images (sample – dark-field / background – dark-field). After the light noise suppression in the resulting images, they were vertically stitched to form a full-sized image. Finally a CT reconstruction algorithm was applied to the array of projections to convert it into a stack of horizontal cross-sections of the sample. The reconstruction also included phase retrieval and a ring artefact suppression filter as necessary. Finally, 3D rendering and visualisation was performed. All steps were performed using the MASSIVE super-computer cluster (Multi-Modal Australian ScienceS Imaging and Visualisation Environment; National Computational Infrastructure, Australian National University, Australia); https://www.massive.org.au). The software used in the pipeline included several open-source projects: ImageMagick (https://www.imagemagick.org) for noise removal, cropping and image format conversion, and CTas (https://github.com/antonmx/ctas) for the removal and stitching of background and dark-field images. Cross-sectional images were reconstructed by the (Linear-Ramp) Filtered Back-Projection algorithm in the XLI-XTRACT software. The reconstructed slices were stored as 8-bit TIFF volumetric images. Images were then rendered using Drishti (version 2.4 or 2.6, github.com/AjayLimaye/drishti).[30] Three dimensional visualisation and manipulation of whole kidneys were achieved using both Drishti and Avizo (VSG Inc., Burlington, MA, USA). ImageJ, Drishti and Avizo were accessed via the MASSIVE facility.

Selection of artery-vein pairs for quantifying shrinkage of Microfil® in rat kidneys

The shrinkage of Microfil® over a period of 24 hours, between the first and second scan, was quantified by a person blinded to the time at which the scan was taken. The widths of artery-vein pairs were measured using Drishti and Avizo (MASSIVE network). Using Drishti, the measurements were taken at the same region in each reconstruction: at the base of branching of vessels (i.e. just prior to the branching of vessels). In total, 244 branching junctions were located in 6 kidneys. Using Avizo, a smaller subset of vessels was assessed by only measuring the diameters of arteries. Two arterial 'lines' were analysed in each kidney, one from the middle and one from the pole of the kidney. This resulted in a total of 66 measurements for this approach.

Sampling of renal tissue for light microscopy

For the rat kidneys, three kidneys were used to assess whether radial geometry of the cortical vasculature determined by micro-CT is similar to that determined by light microscopy. The selected three kidneys were from the first round of beam-time. These three kidneys included curing agent in the Microfil® preparation. Once micro-CT images were obtained, the kidneys were removed from the vials for processing for light microscopy. Each kidney was first cut in the sagittal plane, leaving two halves. One half was then cut in the longitudinal plane. For each plane, 1-2 mm thick slices from two different areas of the kidney were obtained: a slice near the midline and a slice in an intermediate position between the midline and the distal edge of the kidney. A total of 12 slices (four from each kidney) were used for the qualitative morphometric analyses.

For the rabbit kidneys, one half of each kidney was randomly chosen from which two 3 mm slices were taken from the transverse plane. As for the approach taken with the rat kidney, one slice was taken near the midline and one slice was taken from an intermediate position between the midline and the distal edge of the kidney. These slices were then cut in half along the coronal plane so that they could fit on a microscope slide. The remaining half of each rabbit kidney had two 3 mm slices taken from the sagittal plane. Again, one slice was taken near the midline and one slice was taken from an intermediate position between the midline and the edge of the kidney. These slices were then cut in quarters to fit on a microscope slide.

Following overnight immersion in fixative, both rat and rabbit kidney slices were then subjected to two 70% v/v ethanol washes. The slices were then placed in a final rinse of 70% v/v ethanol overnight. They were then dehydrated by immersion in 100% v/v ethanol for 2 h thrice, and then 100% v/v butanol for 2 h before being immersed in 100% v/v butanol overnight. The tissues were then immersed in infiltration solution (Technovit 7100; Heraeus Kulzer GmbH, Wehrheim, Germany) for 2-3 days before being embedded in glycol methacrylate (Technovit 7100; Heraeus Kulzer GmbH, Wehrheim, Germany), sectioned at 3 μm, and stained with haematoxylin and eosin. Sections were scanned with Aperio Scan Scope (Aperio, Vista, CA, USA), providing a digital representation of the entire section.

Qualitative analysis: matching histological sections with corresponding micro-CT slices

ImageScope and Avizo were used to match histological and micro-CT sections of the rat and rabbit. With the histological section viewed on ImageScope (version 11.2.0.780, Aperio Technologies), and the micro-CT 3D volume viewed concurrently on Avizo, large distinctive vessel pairs were first identified on the histological section. Large vessel pairs were of interest because their intimate spatial association may facilitate the diffusional shunting of oxygen.[4] By knowing the approximate location where the histological section was derived, a 2D slice was manually moved through the 3D volume to first obtain an approximate match. Once this was achieved, this 2D plane of section was further adjusted to visualise the same distinctive vessels and any nearby glomeruli that could be identified on the histological section.

Quantitative analysis: comparison of vascular geometry in rat kidneys generated by micro-CT and light microscopy

Only the kidneys of two rats were used in the quantitative analysis on the basis that the analysis in rat kidneys should translate to rabbit kidneys. Furthermore, for this analysis, the experimental unit was considered to be an artery-vein pair rather than an experimental animal. Arterial diameter (shortest length), diffusion distance (shortest distance between the arterial and venous lumen), and proportion of geometric wrapping (proportion of the arterial lumen surrounded by the adjacent venous lumen) were manually measured, for vessel pairs that were matched in both the light micrographs and micro-CT images. Measurements were first made from the light micrographs. Then, measurements from micro-CT images were made a number of days later, without knowledge of the values generated from the light micrographs. A total of 25 matched vessel pairs were identified across the two kidney volumes.

Statistical methods

Data are presented as mean ± standard error of the mean (SEM) unless stated otherwise. Ordinary least products regression was used to generate lines of best fit.[19] Regression analyses were performed using SYSTAT v.13 (Systat Inc, San Jose, CA, USA). Paired t-tests were performed for matched data using Microsoft Excel. For all comparisons, two-tailed $P \leq 0.05$ was considered statistically significant.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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Fig. 1. Micro-computed tomographic images of rat kidneys in which the vasculature was filled with Microfil® with and without curing agent. Images in the left hand panel are scans of whole rat kidneys with Microfil® excluding curing agent. Images in the right hand panel are scans of whole rat kidneys with Microfil® including the curing agent. 'Immediate' represents scans immediately upon tissue preparation, and '24 h' represents scans taken 24 hours following the first scan. Images were generated using Drishti volume rendering software.

Fig. 2. Three-dimensional reconstruction of a whole rabbit kidney generated by synchrotron radiation micro-computed tomography. A reconstruction generated using Avizo. Note the cortical circulation of the rabbit kidney can be visualised up to the level of cortical radial arteries.

Fig. 3. Quantification of the shrinkage of Microfil® over the 24 hours after sample preparation. (A) Measurements of paired vessels (n = 244) immediately after ('Immediate') and 24 hours after ('24 h after') tissue preparation. **(B)** Measurements of arterial diameter only (n = 66). **(C)** Scatter-plot of paired vessel diameter immediately and 24 hours after sample preparation. **(D)** Scatter-plot of arterial diameter immediately and 24 hours after sample preparation. In (A) and (B), columns and error bars represent mean ± standard error of the mean. * $P \leq 0.05$, derived from Student's paired t-test. Lines of best fit in panels (C) and (D) were generated by ordinary least products regression.[19]

Fig. 4. Images of the kidneys of rats generated by light microscopy and by micro-computed tomography. Left and right pairs of images show sections in the transverse and longitudinal plane respectively. Histological images were stained with haematoxylin and eosin and photographed at 0.7× (A & K), 2× (I), 3× (Q), 4× (C, E, G, M, O), or 5× (S) magnification. Objects identified by coloured circles in A, B, K & L are shown at higher magnification in the lower panels. Coloured circles in the images shown at higher magnification identify glomeruli matched in both micro-CT and histological images. A = artery, V = vein, * = absence of tissue, thus appears black in micro-computed tomographic images. Note the presence of Microfil® within blood vessels in the stained histological sections, some of which has been affected by shrinkage and dislodged during processing. Note also the presence of ring effects around arteries and veins in the images generated by micro-CT, arising because the specimen was placed at some distance from the detector in these studies. The outer margin of the ring corresponds to the limit of the lumen of the vessel (see analysis in Fig. 6).

Fig. 5. Images of the kidneys of rabbits generated by light microscopy and micro-computed tomography. Histological images in the transverse plane were stained with haematoxylin and eosin. Objects identified by the yellow boxes in **A-D** are shown at higher magnification in the lower panels **(E-H)**. A = artery, V = vein, * = absence of tissue, which appears black in micro-computed tomographic images. Other matched arteries and veins in the section are indicated by red and blue arrows respectively. Note the absence of ring effects in the images generated by micro-CT, which were avoided by placing the specimen close to the detector. Histological images A, C, E, and G are at magnifications 0.3×, 0.4×, 2×, and 1.2× respectively.

Fig. 6. Relationships between measures of radial vascular geometry in rat kidneys determined by light microscopy and micro-computed tomography (micro-CT). (A) Scatter-plot of arterial diameter. **(B)** Scatter-plot of diffusion distance. **(C)** Scatter-plot of proportion of wrapping (proportion of the wall of the artery in contact with the paired vein). Lines of best fit were determined by ordinary least products regression.[19] Note the close agreement between measures generated by light microscopy and micro-CT when the edge effect in the micro-CT images was included in the measurement.