Three-dimensional imaging of vasculature and parenchyma in intact rodent organs with X-ray micro-CT

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Jorgensen, Steven M., Omer Demirkaya, and Erik L. Ritman. Three-dimensional imaging of vasculature and parenchyma in intact rodent organs with X-ray micro-CT. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1103–H1114, 1998.—A microcomputed tomography (micro-CT) scanner, which generates three-dimensional (3-D) images consisting of up to a billion cubic voxels, each 5–25 µm on a side, and which has isotropic spatial resolution, is described. Its main components are a spectroscopic X-ray source that produces selectable primary emission peaks at ~9, 18, or 25 keV and a fluorescing thin crystal plate that is imaged (at selectable magnification) with a lens onto a 2.5 × 2.5-cm, 1,024 × 1,024-pixel, charge-coupled device (CCD) detector array. The specimen is positioned close to the crystal and is rotated in 721 equiangular steps around 360° between each X-ray exposure and its CCD recording. Tomographic reconstruction algorithms, applied to these recorded images, are used to generate 3-D images of the specimen. The system is used to scan isolated, intact, fixed rodent organs (e.g., heart or kidney) with the image contrast of vessel lumens enhanced with contrast medium. 3-D image display and analysis are used to address physiological questions about the internal structure-to-function relationships of the organs.

microvasculature; nephron; muscle fiber; branching geometry; basic functional unit; microcomputed tomography

SEVERAL ASPECTS of organ structure can have profound impact on the function of the organ. Examples relevant to the circulation include 1) the opposing effect of factors increasing the efficacy of a vascular tree in terms of energy needed to fabricate and operate the tree relative to the proportion of the organ occupied by the vascular tree; 2) the influences of the orientation and interconnection of the muscle fibers and the intermuscle fiber stroma on the mechanical efficacy of the heart wall; and 3) the detailed intertwining of the postglomerular blood vessels and the renal tubules on renal excretory function. These examples have in common the question as to how the packing, interconnectivity, perfusion, or drainage of an organ’s basic functional unit (BFU; the smallest integrated assembly of diverse cells that functions like an organ, e.g., the nephron and the hepatic lobule, which are generally of the order of 0.1 mm³ in volume) impacts on the interaction (especially structural) of the BFUs within an organ. This information must be known if the functional characteristics of the BFUs [increasingly measured in terms of the function of its component cell(s) by molecular biological methods] are to be translated into the integrated organ function. This interaction is complex due to the sheer number of BFUs within an organ, the fact that each BFU’s function can be modulated independently, and because BFUs can impact on each other structurally and functionally.

We believe that three-dimensional (3-D) imaging provides the ability to quantitate the independent BFUs and their physical locations and orientations relative to each other, which are factors that are needed to address the physiological questions posed above. Such a 3-D image will need to resolve individual BFUs and to simultaneously detect and measure all BFUs within an entire organ (or at least a meaningful portion of it, such as a lobe of the organ). This means that, for mice and rats, we need to image a volume of up to 2 cm³ with at least 10 µm, isotropic resolution.

The methodology described here is an attempt at addressing the need for linking our rapidly increasing knowledge about the expression of genes (in cells) to the structure and function of the whole organ (9). Because much of our detailed knowledge of genes and our ability to control gene expression is available in mice and rats, the ability to quantitate the impact of highly targeted genetic manipulations on organ function would appear to be a step in the direction of answering the question as to how genes link to whole body pathophysiology. The ability to effectively detect and quantitate individual BFUs, quantitate their common mechanical linkage, blood supply, and “drainage” trees, as well as quantitate their packing within an intact organ has the potential for contributing to our understanding the “emergent function” of these huge assemblies of interacting BFUs (26).

METHODS

Microcomputed Tomography System Hardware

Our microtomographic system design (Fig. 1) is based on a microcomputed tomography (micro-CT) scanner developed (primarily for quantitating microscopic pore structure within rock specimens) by Flannery et al. (13) of EXXON Research and Engineering (Annandale, NJ). The following is a descrip-
The transmitted X-rays are converted to visible light by a fluorescent scintillating crystal plate (scintillator) of cesium iodide doped with thallium [CsI(Tl)]. This crystal generates ~1,000 light photons per absorbed 18-keV X-ray photon. The 1-mm-thick 4.5-g/cm² crystal absorbs essentially all 18-keV X-rays. The CsI(Tl) plate is covered with blackened aluminum on the X-ray side to minimize the light reflections at the front surface of the crystal. Although this cuts in half the light that we can detect, it is essential for preventing the loss of spatial resolution that would result from the light internally reflected from the X-ray source side of the crystal. The CCD side of the crystal is covered with a sapphire plate that is coupled to the CsI(Tl) with silicon oil and is transparent to light but provides a barrier to the moisture in ambient air. The light image generated within the CsI(Tl) crystal plate is in turn transferred by a lens onto the surface of a CCD-based imaging camera. This flat field lens currently consists of an f/2.8 enlarger objective for 1- to 10-fold magnification. Thus, if the magnification is twofold, a 1-cm² X-ray image is imaged by a 2-cm² area on the CCD chip, effectively making the CCD pixels 12 μm on a side (referenced to specimen). The use of a coupling lens is much less efficient in converting X-ray scintillations into a signal from the CCD than a fiber-optic coupling. However, the lens allows us to conveniently alter the magnification of the fluorescent image, a flexibility that we need while we establish the imaging needs for different organs and scientific questions.

Step 4. The continuous illumination pattern generated within the clear CsI(Tl) crystal is converted into a discretized pattern of stored charge in the CCD’s (SiTe CCD 1024 TKB) pixels. The charge in each of the 1,024 × 1,024, 24 μm on-a-side, square pixels is digitized and stored as an array in a computer until 721 projections, at small increments of specimen rotation around 360°, have been acquired. The actual number of projections needed is governed in large measure by the tomographic resolution desired (8), although the imaged slice thickness cannot be less than the CCD pixel “height.” The CCD camera is cooled, both by chilled water and thermoelectrically, to ~40°C so as to reduce thermal noise in the signal. The CCD dark current at ~40°C is 1 electron·s⁻¹·pixel⁻¹. The CCD is backside thinned and illuminated, coated with antireflective coating, is ~80% quantum efficient to the 550-nm light generated by CsI(Tl), and can be read out at up to 500,000 pixels per second while maintaining the 16-bit gray scale fidelity.

Step 5. The 16-bit analog-to-digital (A/D) converter has been set up so that each of the A/D conversion steps is equivalent to five electrons, which is approximately equivalent to the readout noise of the CCD. With the CCD’s nominal full-well capacity of 450,000 electrons, this means that the medium 16-bit A/D value is reached at ~75% of the nominal full-well condition. This ensures that we utilize the most linear portion of the light input-to-electronic signal output transfer curve and avoid nonlinearity issues in individual CCD pixels, which may fall short of the normal full-well capacity. The CCD chip readout operation is via a controller (ST138 Princeton Instruments CCD camera controller) that is programmed to alter binning, region of interest (ROI), A/D conversion rate, and depth and integration time.

Step 6. The entire camera is suspended from a goniometric cradle (BG 120MS) that has 0.01° readability, 0.001° step size, and 0.05° accuracy. This rotation capability greatly facilitates alignment of the CCD chip array with the axis of rotation of the specimen. The entire imaging “chain” (i.e., scintillator, lens, CCD camera) is attached to a linear transla-
Fig. 2. Photographs of our bench top microcomputed tomography (micro-CT) scanner. A: general view of the micro-CT scanner. A "floating" table isolates the scanner from the vibrations in the floor, and a lead-lined box surrounding the scanner shields personnel from scattered X-rays. On the left is the spectroscopy X-ray source. The collimated X-ray beam is shielded by a lead-wrapped brass tube as the beam passes from the source into the shielded cover of the scanner. A personal computer (right) is used to control the scanner and to store the CCD image data recorded at each angle of view during the scan. B: detailed view of the scanner within the shielded enclosure. See text for details of components.
operation stage (MT 160) so that the distance of the imaging chain from the specimen rotation axis can be controlled. The position of the scintillator with respect to the lens, along the optical axis, can be independently adjusted (UT-5020PP) with 0.1 µm resolution so as to permit fine focusing adjustment of the fluorescent image onto the CCD chip. The entire imaging chain is suspended from a bridging support stand (Newport/ Klinger X95 Structural Rails).

Operation of the Micro-CT Scanner and Image Preprocessing

The coordinated operation of the scanner specimen positioning system (automated focus, rotation, and translation stages), the image acquisition system (camera), and initial storage system (local digital disks) is provided by an enhanced Pentium-based personal computer.

The processing of the projection data and subsequent CT image reconstruction is currently performed with a Silicon Graphics Origin 2000 computer. For the X-ray CT application, at each angle of view, the system records an array of pixel gray scale values \( I(x,y) \). Every 10 or so angles of view the specimen is removed from the X-ray beam and the raw beam is recorded \( I(0,x,y) \). Note that, strictly speaking, \( I(0) \) is a function of time, and not of \( t \), but, for convenience, we use \( t \) as a time reference as our scanning is done sequentially with specimen angle correlated to elapsed time. All of these images first have an averaged “image” of no exposure \( I_{ABL}(x,y) \) subtracted, and the final signal(s) is calculated as \( S(0,x,y) = \text{log}_2[I(0,x,y) - I_{ABL}(x,y)] - \text{log}_2[I(0,x,y) - I_{ABL}(x,y)] \). \( I_{ABL}(x,y) \) is assumed to change insignificantly during the scan sequence; hence, only one value needs to be used. \( I(0,x,y) \) changes slowly with time (hence with \( t \)), so that the average value allows for adequate compensation for the gradual decrease in \( I_{0} \) over time. The duration of a scan depends on the object diameter, its predominant material content, and resolution desired because these factors impact on the duration of X-ray exposure at each angle and number of angles of view needed. Typically, a scan of a 2-cm-diameter soft tissue specimen, using 360 angles of view, requires 11 h.

Tomographic Image Reconstruction

The X-ray projection data covering the entire volume scanned are submitted to the reconstruction program, resulting in a volume image of up to 10,243 voxels, each cubic voxel being 5–25 µm on a side, depending on how much of the specimen is imaged.

Backprojection. Because we use an X-ray focal spot that subtends \( <0.8 \) mrad at the scintillator, we have reasonably pure cone-beam geometry. In addition, when the scanned volume within the specimen subtends an angle at the X-ray focal spot that is less than the angle subtended by the CCD pixel height divided by the diameter of the scanned volume, we can reasonably assume that the cone-beam geometry is well represented by a stack of contiguous, parallel, transverse fans that ignore the axial divergence of the X-ray beam. This greatly simplifies the backprojection process. In general, however, we use a cone-beam backprojection, modified Feldkamp reconstruction algorithm (32).

Global transaxial image reconstruction. The conventional convolution-based CT reconstruction method (32) requires that all of the line integrals through a single point on a tomographic plane are measured. That implies that the scanner images the entire transaxial extent of the object scanned at every angle of view. An example of a global reconstruction of a single cross section is provided by Fig. 3.

ROI reconstruction. A region within the object can be imaged selectively by several methods. We have used the following methods (27) for dealing with missing or incomplete sets of scan data:

**ROI Measurement Without Acknowledging the Out-of-View Geometry of the Specimen.** The incomplete data set (as far as the data needs for global reconstruction are concerned) makes the global reconstruction algorithm quantitatively invalid. As shown in Fig. 4, right, global reconstruction of the ROI scanned results in a concentric gray scale shading that becomes progressively brighter radially outward.

**Use of Profile-Extension Based on Knowledge of the Out-of-View Geometry of the Specimen.** This method requires knowledge of the out-of-field-of-view geometry of the scanned object. For an object such as a heart, which has an elliptical cross section and is of fairly uniform radiopacity, this method works quite well. If, however, it is of irregular or unknown shape and/or of heterogeneous opacity such as trabecular bone, then streak artifacts occur that could greatly impact the imaged ROI.

**Local Reconstruction.** To overcome some of the problems associated with unknown shape of the out-of-field-of-view specimen and heterogeneity of radiopacity, we use a local reconstruction algorithm (34). Local tomography is “local” in that reconstruction at a point requires integrals only over lines passing infinitesimally close to the point, and the local reconstruction produces the same smooth regions and boundaries as the global reconstruction. Local reconstruction algorithms allow for scanning objects larger than the X-ray detector area because they only require that the 3-D ROI is always within the field of view imaged. If contiguous small volumes are scanned with accurate registration made possible by the great precision of the mechanical stages, large volumes can be scanned piecewise with spatial resolution higher than the CCD imaging array could allow if it were used to image the entire organ at one time. Local reconstruction does, however, have the limitation that absolute attenua-
nation values (i.e., gray scale) for each reconstructed voxel are lost. Differences in absolute attenuation values between adjacent structures can, however, be estimated from the local reconstruction image.

Deblurring. Blurring of the X-ray image as recorded by the CCD-based images is due to several factors. These include the X-ray focal spot, which causes some penumbral blurring; another is light diffusion within the fluorescent crystal; and another is the optical transfer function of the lens imaging the fluorescent screen onto the CCD camera chip. These blurring influences can be reversed somewhat by a constrained iterative deconvolution method (1). A “knife edge” image is used to generate the point spread function of the imaging system used in this deconvolution. From the Fourier transform of the derivative of the imaged knife edge we also obtain an estimate of the system’s modulation transfer function (19). In both approaches, we tried time and spatial averaging of the imaged knife edge using the method described by Boone et al. (6). Without “sharpening” of the projection image, at 10% modulation and at 1:1 optical magnification, the spatial resolution of the CCD generated image was measured to be 20 line pairs per millimeter; and at 4:1 optical magnification it is 48 line pairs per millimeter. After deconvolution with the imaging system point spread function the value becomes 56 line pairs per millimeter at the 4:1 magnification.

Ring artifact removal. In addition to sharpening the images by image preprocessing, additional processing of the projection images before the tomographic image reconstruction process can help to overcome the ring artifacts resulting from fixed patterns in the recorded projection images (23). These fixed patterns are primarily due to incompletely calibrated sensors in the detector system and small localized defects in the fluorescent crystal plate. These blemishes result in ring artifacts in the tomographic images because only a single detector array is used to acquire the projection data from all angles of view about the specimen (i.e., a 3rd-generation CT scanner architecture). These artifacts are undesirable because they may interfere with the qualitative and quantitative analysis of images.

CT image segmentation. Contrast features in the CT images (e.g., radiologically opacified microvessels) must be segmented objectively, reproducibly, and accurately, conditions best achieved with automated image analysis techniques. One such method is based on a minimum error thresholding algorithm (22). This algorithm assumes that the frequency histogram of a 3-D CT image gray scale value consists of two normally distributed populations (background and object of interest) with unequal variances. The thresholding algorithm uses a criterion function that reflects the amount of overlap (i.e., classification ambiguity) between the Gaussian models of the background and object-of-interest gray level intensity distributions. The algorithm sequentially searches through the histogram for the gray level intensity at which the criterion function is minimum.

RESULTS

Resolution of the Tomographic Images

For evaluation of the effective resolution of the 3-D images, we devised a 1-cm-diameter cylinder of plastic in which small soda-lime-glass beads (nominally 300, 100, 30, and 10 µm diameter) were suspended at (18 keV, the published X-ray attenuation coefficient of soda-lime-glass is ~7.8 cm⁻¹ and that of water is 1.1 cm⁻¹). Figure 5 is a volume-rendered display of a 500-µm-thick slab through the diameter of the test phantom. Figure 6 shows profiles of reconstructed densities through diameters of the 100-, 30-, and 10-µm-diameter microspheres in thin tomographic images generated using global reconstruction. The profiles show a slight rounding off of what would ideally be square-wave profiles. The precision with which an index of the glass beads X-ray attenuation coefficient can be estimated from these images is indicated by the fact, for 100-µm-diameter beads, the value is 1.01 of the value observed in 300-µm beads; for 30-µm-diameter beads, it is 0.37, and for 10-µm beads it is 0.14.

Because the voxel size is 6 µm in this analysis, dimensional analysis of the images of the 10-µm-diameter microspheres was not pursued. Nondimensional estimates of those small microspheres can, however, be made with the brightness area product method (5) in which case our estimated diameter was 16 µm.

Table 1 summarizes the accuracy of our estimates of microsphere volume and the role of the axial and radial...
locations of the microspheres in the scanned volume. We expect there to be increased distortion of the microsphere in the axial direction the further the sphere is away from the central plane of symmetry of the X-ray cone-beam. Similarly, the radial distance from the axis of rotation also has an impact on the accuracy because the peripheral zone of the CT image suffers from reduction of the number of transmitted rays passing through each voxel; hence, a “noisier” image is to be expected. These estimates were made from a scan involving 721 equispaced angles of view around 360°. If smaller numbers of views are used (thereby speeding up the scanning process), the estimated volumes are progressively underestimated as the number of angles is decreased. This effect is proportionately greatest for the smallest microspheres.

Examples of 3-D Imaging Capability of the Cardiovascular System

 Coronary vascular tree. Figure 7 illustrates the sort of quantitative information obtainable from our 3-D

<table>
<thead>
<tr>
<th>Microsphere Diameter, µm (True Volume)</th>
<th>Microsphere Volume From CT Image</th>
<th>Radial Zones</th>
<th>Microsphere Volume From CT Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>31.9 (16.9±0.4)</td>
<td>A1 17.3±0.7 (36)</td>
<td>R1 15.7±0.4 (108)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A2 16.1±0.6 (38)</td>
<td>R2 18.9±0.6* (49)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A3 13.9±0.6 (38)</td>
<td>R3 22.4±0.1 (20)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>A1 603.0±2.2 (11)</td>
<td>R1 596.8±12.5 (35)</td>
<td></td>
</tr>
<tr>
<td>(520.0±8.2)</td>
<td>A2 584.1±2.2* (9)</td>
<td>R2 619.9±22.5 (16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A3 513.4±1.8* (7)</td>
<td>R3 654.6±18.8* (15)</td>
<td></td>
</tr>
<tr>
<td>278</td>
<td>A1 12.76±336 (4)</td>
<td>R1 12.13±311 (19)</td>
<td></td>
</tr>
<tr>
<td>(11,240±1,038)</td>
<td>A2 11.289±335* (6)</td>
<td>R2 12.141±741 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A3 12.702±341 (7)</td>
<td>R3 12.251±342 (3)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of microspheres in zone. 3D, three dimensional; CT, computed tomography. Axial zones were 1,024 µm high, radial zones were 990 mm wide, and volumes were 1,000 µm³. *Mean microsphere volumes that were significantly (P < 0.05) different from microsphere volumes in A1 or R1. †Mean volumes that were significantly (P < 0.05) different from the true volumes.

Fig. 5. Volume-rendered display of 500-µm-thick slab through diameter of the cylindrical test phantom used to evaluate 3-D distribution of spatial resolution (see Table 1). Only the image data with gray scale exceeding the automatically determined gray scale threshold, discriminating the microspheres from the background, are displayed. Lower edge coincides with the central plane of the X-ray cone-beam, and the axis of rotation passes vertically through the center of the image. With 6-µm cubic voxels, the 10-µm microspheres show as barely detectable specks of “noise.” The 300-, 100-, and 30-µm diameter glass microspheres are clearly visible and their shape discernible.

Fig. 6. Tomographic images of glass microspheres suspended in a plastic cylinder. These images were computed from the stack of transaxial images generated by the scanner so that they are oriented in the axial plane at right angles to the transaxial plane. The X-ray CT number (i.e., image brightness) values along diameters of different diameter microspheres are plotted below each image. Ideally these profiles are square waves, but several features of the CT scanner and reconstruction process blur the edges of objects and thereby round off the profiles.
image CT scanner. Optimization of vascular branching geometry has been studied by many investigators over the past 70 years, but all have been handicapped by the great practical difficulty of measuring the blood vessels (36). Our 3-D digital images should now be amenable to largely automated analysis (17). Lack of appropriate change in blood supply architecture is possibly an important contributor to reduced efficiency of myocardial function in acquired myocardial hypertrophy (2). The extent to which the arterial growth does not provide the functional reserve capability seen in nonhypertrophied myocardium may also be due to some of the muscle fibers becoming mechanically disadvantaged and therefore in need of more blood flow than in the normal myocardium.

Figure 8 illustrates the ability to isolate individual vessels from the complex network perfusing a block of porcine heart wall and the ability to quantitatively analyze the vessel dimensions. This analysis is performed with a computer program of the sort developed by Higgins et al. (17).

Myocardial muscle fiber "architecture." It is a common observation that almost all tubular muscular organs have helically arranged muscle fibers in the wall. Of particular interest is the muscle fiber architectural arrangement in the myocardium. Figure 9 illustrates the sort of data that can be obtained from the 3-D image data. Examples of hypotheses that "explain" the spiral arrangement of muscle fibers in the myocardium include maximization of a vessel’s lumen volume for minimum length of fibers and that the particular arrangement in the left ventricular wall tends to result in equal stress on the muscle fibers despite their different locations and radii of curvature in the wall (3). Our measurements will be available throughout the entire heart wall; hence, the universality of these hypotheses can be tested, which is something that has been well-nigh impossible to date because of the prohibitive logistic task that would be involved.

Skeletal muscle vasculature. Figure 10 is a display of the 3-D image of a rat hindlimb made with the micro-CT scanner. The blood vessels have been filled with PbO4 suspended in a silicon polymer (Microfil), and these appear, as well as the bone, in the CT images. Detailed information about the diameters and lengths of the connected vascular segments and the branching angles between the “mother” and “daughter” branches would allow us to perform quantitative evaluation of...
the distribution of vascular resistances and how this changes in the presence of vascular remodeling. In addition, we could evaluate how this branching geometry might impact on the spatial heterogeneity of perfusion of the muscle.

Coronary artery vasa vasorum. Figure 11 is an example of our ability to visualize the 3-D anatomy of vasa vasorum in a section of porcine coronary arterial wall. A question of interest relates to the role of angiogenesis in neointimal formation after coronary artery injury (24). The micro-CT images are uniquely suited not only to quantitate angiogenesis within the arterial wall but also to integrate arterial structure and vascular biology through 3-D image data combined with molecular biological methods using immunohistochemical methods applied to histological sections of the same specimens after completion of the CT scan. Preliminary analyses of this type (25) show marked spatial disorganization and increased spatial density of coronary vasa vasorum after intracoronary artery balloon dilatation.

Renal vascular system. Figures 12 and 13 illustrate the type of image data that we acquire with micro-CT of a rat kidney. The 3-D microstructure visualized with these images is important for reasons such as in the following hypothetical example. The efferent arterioles of the juxtamedullary nephrons branch into a “horse-tail” bundle of capillaries. They are arranged as a parallel set of capillaries of increasing length. If vascular resistance of these parallel branches are equal per unit length then the longer vessels will receive less flow than the shorter. If the pressure head is increased, more flow will occur through the longer vessels with the additional consequence that more of the papillary medulla is perfused with subsequent differences in solute exchange consistent with the extravascular milieu changes down toward the papilla. This is an example where knowledge of the vessel function is insufficient, because the location of the vessel is of critical importance. Now, if the kidney grows in size in response to a uninephrectomy (20), the anatomic proportions may change sufficiently so that the position-related aspect of the function could change. As an example of this methodology, Ortiz et al. (31) have shown that cortical microvasculature is markedly affected in rats subjected to experimental hepatic cirrhosis.

**DISCUSSION**

Performance Relative to Biological Measurement Needs

As indicated in Table 1, the error with which we can estimate the volume of the glass beads (as a fraction of the true volume) decreases with increased diameter. This error is attributable to a number of factors such as the fact that the 3-D image is made up of discrete voxels (11, 14), that there is noise in the gray scale values due to X-ray photon statistics, and other imaging factors (8) due to blurring of the original X-ray projection image recorded by the CCD imaging array, due to the X-ray focal spot's finite size, the thickness of the fluorescent
crystal, and the optical limitations of the lens that projects the image onto the CCD array. In addition, the CT image reconstruction algorithm (12) also distorts the image, especially in the axial direction due to the cone-beam geometry of the X-ray beam. These factors are all interrelated.

Performance Relative to Other X-Ray Micro 3-D Imaging Methods

Scanner design. Our approach uses high-resolution electro-optical imaging of the X-ray image of a specimen held close to the imager. This approach has several advantages over the more traditional use of X-ray magnification in which the specimen is held close to the X-ray focal spot so that a large X-ray image is projected onto a large area-imaging device such as an image intensifier (10, 30). Advantages include reduced blurring due to the size and shape of the X-ray focal spot and reduced cone-beam angle and therefore reduced artifacts associated with cone-beam reconstruction algorithms. The approach that we have pursued is now possible because of several state-of-the-art hardware developments such as the large-array CCD chips. In addition, the commercial availability of large-memory-capacity computer hardware that can accommodate the huge 3-D images (e.g., 10,243 voxels) and compute-intensive algorithms required to produce and analyze high-resolution, large-volume images is also important. However, although CT scanning a volume the size of an intact rodent organ at near cellular resolution is in part a matter of downsampling a clinical whole body CT scanner, it requires accommodating several physics aspects that can be largely ignored with whole body CT. A very different approach to micro X-ray imaging is to use zone-plate X-ray optics to encode the X-ray 3-D image data rather than doing a rotational scan (18). Although this approach has had some success with low-energy X-rays and rather small specimens, it is unlikely to be suitable for 3-D imaging of specimens the size of intact rodent organs.

In regard to relative costs of the various 3-D microimaging approaches that are available, the cost of the components used in our system was less than $150,000.

X-ray source. X-ray microscopic 3-D imaging depends importantly on the characteristics of the X-ray source.
The synchrotron (29) provides a nearly ideal source in that it produces a very intense beam of electromagnetic radiation so that selection of a narrow bandwidth, essentially monochromatic X-ray beam is still sufficiently intense to permit X-ray imaging with adequate photon statistics within a reasonable (biologically speaking) period of time (21). In addition, the mean wavelength of the near monochromatic beam can be tuned to optimally accommodate the thickness of the specimen. Finally, the X-ray beam is very close to parallel, which permits use of a parallel-beam CT image reconstruction algorithm that greatly reduces the computational burden. However, synchrotron X-ray sources are not without limitations. They include the fact that very few beam lines are available and those that exist are available only intermittently for this purpose. Also, sophisticated X-ray optical devices are needed to spread the beam to accommodate specimens greater than ~1 cm diameter if mechanical transverse scanning is to be avoided. Nonetheless, because of these salutory characteristics, synchrotron-based micro-CT scanners will remain important for continued development and evaluation of new techniques applied to micro-CT approaches.

Bench top quasi-monochromatic X-ray sources such as the one we use are available, but other methods are under development at present. These sources all suffer in that the X-ray intensity is limited, resulting in greatly increased duration of imaging. The X-ray generation approach that we have taken appears to be the best compromise at present, although it will be succeeded when some of the new techniques are implemented on a routine basis.

X-ray and matter interaction for image contrast generation. Although we use the common method based on X-ray attenuation as the source of image contrast, there are several other uses of X-ray in micro-CT that utilize different aspects of the interaction of X-ray and matter. One such approach is to use phase contrast (i.e., measure different time delays of X-ray propagation through different materials). Although phase contrast has much higher inherent contrast in tissues than does attenuation contrast (4), this method is as yet very much in the early development stages and involves a complex, lengthy scanning and signal analysis process. Another approach is the detection of angle-dependent scatter of the X-ray by the different tissues. This X-ray diffraction tomography has potential for differentiating molecules that are made up of the same atoms, but the molecular arrangement of these atoms differs sufficiently to result in different diffraction "signatures" (15). Another approach uses the emission of fluorescent X-radiation from selected atoms purposely introduced into the specimen (35). If the illuminating X-ray energy is precisely known and of narrow bandwidth, and the fluorescence of the "contrast" atoms is also of known wavelength, the Compton and Thompson scattered X-rays (broad band) can be excluded from the measurement by using a high-purity germanium detector.

Specimen damage by X-ray exposure. The overall limitation of X-ray imaging is the damage caused by the X-rays (28). One limitation is set by the damage of critical proteins that would ultimately kill the specimen. As our current approach uses fixed specimens, the limit is set by the heating caused by the radiation. This limit can be exceeded with synchrotron radiation but not with the spectroscopy source at the focal spot-to-specimen distance we use. We estimate the exposure of our specimens during a complete scan to be ~104 rad. Although this does not noticeably damage our specimens, it could cause problems for in vivo, longitudinal studies. This, in addition to needing to speed up the scan so as to minimize motion blurring, is another reason to reduce the spatial resolution of a scan in longitudinal studies.

Performance Relative to Other, Non-X-Ray Micro-3-D Imaging Methods

Light microscopy. Light microscopy can also be used to generate 3-D images. One method is serial sectioning for generating the images of multiple thin slices cut from a specimen. The problem with this approach is the difficulty in registration of the adjacent slices and distortions that occur during the sectioning process. The main limitation, however, is that this mechanical process is slow and presents a considerable logistic difficulty. Most importantly, once sliced, the intact volume is lost so that any evaluation of the volume by other methods is made very difficult if not impossible.

Confocal microscopy. Confocal microscopy was developed primarily to reduce the loss of contrast due to the contiguous tissue that is blurred and mixed with the image of the slice of interest within an intact volume of tissue. This approach is used to also scan sequential slices with the advantage that the volume is not physically sliced. Practically speaking, the largest volumes that are imaged with confocal imaging (including 2 photon) are generally <1 mm3 so that continuous measurements are not possible in an intact organ ~1 cm3 in volume.

Scanning electron microscopy. Scanning electron microscopy is limited in that it images a projection of the specimen so that the surfaces only are conveyed, which does not provide the 3-D information we seek.

Micromagnetic resonance imaging. Micromagnetic resonance imaging (micro-MRI) has been successfully used, but the features imaged are quite different from those in X-ray images. Most commonly, MRI images are generated with factors related to the proton mobility. As the resolution of these systems depends directly on the magnetic field strength, special magnets with superconducting coils have been developed to give a microscopic resolution of 10 µm at 7 tesla. Reese et al. (33) have used diffusion-tensor magnetic resonance to image the spatial distribution of myocardial fiber architecture (i.e., angle to the short axis plane). However, that method is limited because it does not provide information about the mechanical interconnection of fibers from plane to plane. Such information is available in the micro-CT 3-D images and could be useful for modeling electrical and mechanical aspects of the myocardium.
Future Development Possibilities

The system described here is limited in its application to isolated fixed specimens. This is due to the limited X-ray flux (i.e., time rate of delivery of X-ray photons per unit solid angle emitted from the source) that can be generated by current bench top X-ray sources and due to the need to detect a certain number of X-ray photons per voxel in the reconstructed image. The higher the number of photons detected the higher the spatial and contrast resolution that is possible. Ultimately, we would like to have sufficient flux to permit scans of sufficiently short duration to permit imaging of in situ organs in living animals. Although the in situ leg (tibia) of anesthetized rats has been scanned in vivo using a synchrotron X-ray source (21), it is unlikely that those in situ organs such as the heart and gut (that cannot be restrained for a sufficiently long period of time to permit a micro-CT scan) will be amenable to this technique in the foreseeable future, even with high flux sources such as synchrotrons. However, with the current system, there are several trade-offs that can be made. For instance, the X-ray source could be positioned closer to the subject (flux is proportional to the square of the distance between specimen and X-ray source), but this would result in increased computation time due to the greater cone-beam angle. Another method is to increase the size of the voxel (i.e., decrease the spatial resolution) so that the total exposure to X-ray can be reduced (proportional to the cube of the voxel side dimension). The utility of these approaches depends on the resolution needed for the study.

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