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Method Article

Iodine-based contrast staining improves micro-computed tomography of atherosclerotic coronary arteries



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A B S T R A C T

We sought to develop a reversible staining protocol using micro-computed tomography (micro-CT) paired with a radiopaque contrast agent that allows for three-dimensional *in situ* visualization and characterization of atherosclerotic plaques. Atherosclerotic porcine coronary arteries were dissected from surrounding myocardium and incubated in iohexol at various concentrations and incubation times and then imaged using direct radiography. Line profiles were generated across the artery x-ray to determine effectiveness of the radiopaque contrast agent to penetrate the tissue. Our studies revealed that, to sufficiently delineate tissue constructs, the minimum effective iohexol concentration and incubation time were 240 mgI/mL for 1 hour. Among all groups, 24 hours of de-staining brought radiopacity back to control levels. After iohexol incubation, micro-CT was performed. Our findings demonstrate that extended staining times and a minimum iohexol concentration of 240 mgI/mL are required for effective tissue perfusion, which eliminates the diffusion distribution profile inherent to the ability of the contrast agent to traverse tissue layers.

- Iohexol enhances *ex vivo* micro-CT imaging of atherosclerotic coronary arteries
- Iohexol allows for improved tissue segmentation during micro-CT image analysis
- Effectiveness of iohexol penetration of the tissue was dependent on concentration and duration of incubation

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Specifications Table

Subject Area	Veterinary Science and Veterinary Medicine
More specific subject area	Cardiovascular Pathology
Method name	<i>Atherosclerotic coronary artery contrast enhancement for micro-CT</i>
Name and reference of original method	N/A
Resource availability	GE Omnipaque (Iohexol), Sylgard 184, stainless steel pins, Centrifuge tube (50-mL conical), X-ray positioning foam, NSI efX50 micro-CT, NSI efX50 reconstruction software, 3D Slicer, ImageJ

Method details

To image soft tissues with micro-CT and subsequently segment tissue layers using 3D image processing software, a contrast agent must be applied prior to micro-CT image acquisition. The following method optimized the application of iohexol in micro-CT imaging of atherosclerotic porcine coronary arteries to determine the conditions required to augment radiopacity of adjacent soft tissues for delineation of tissue type [1].

Isolation of coronary arteries: Porcine conduit coronary arteries (approximately 3–5 cm luminal diameter) were dissected from surrounding tissue using a stereo microscope. The samples were maintained in Krebs bicarbonate buffer (0–4 °C) during dissection and subsequently arteries were submerged and stored in 10% neutral buffered formalin (NBF) for fixation.

Method Optimization: Preliminary testing was performed to determine the stain concentration and incubation time most appropriate for optimized tissue penetration and contrast enhancement. Five atherosclerotic porcine coronary artery samples that had been fixed in 10% NBF were stained with 2.4, 12, 24, 240, and 300 mgI/mL iohexol for 10 min, 30 min, 1 h, 3 h, or 24 h. For preliminary testing, the samples were mounted as shown in Fig. 1. X-ray positioning foam and parafilm sealing film were used to construct an imaging apparatus and supports. Two pieces of foam, each approximately 4 in x

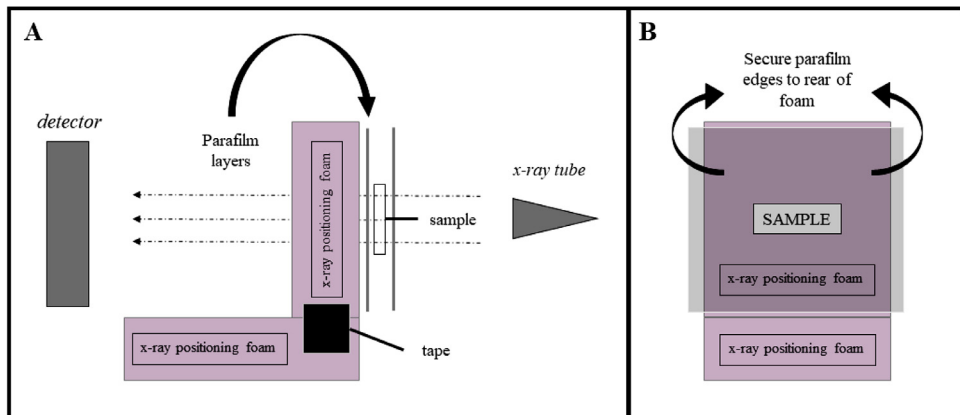


Fig. 1. Schematic of the sample mounting apparatus used for DR image acquisition. Vessel segments, lumens cleared of fluid, were mounted as shown, supported by two layers of parafilm, secured to x-ray positioning foam. Panel A illustrates a right-side view of the mounting rig. Panel B illustrates a front-facing view as seen from the x-ray tube.

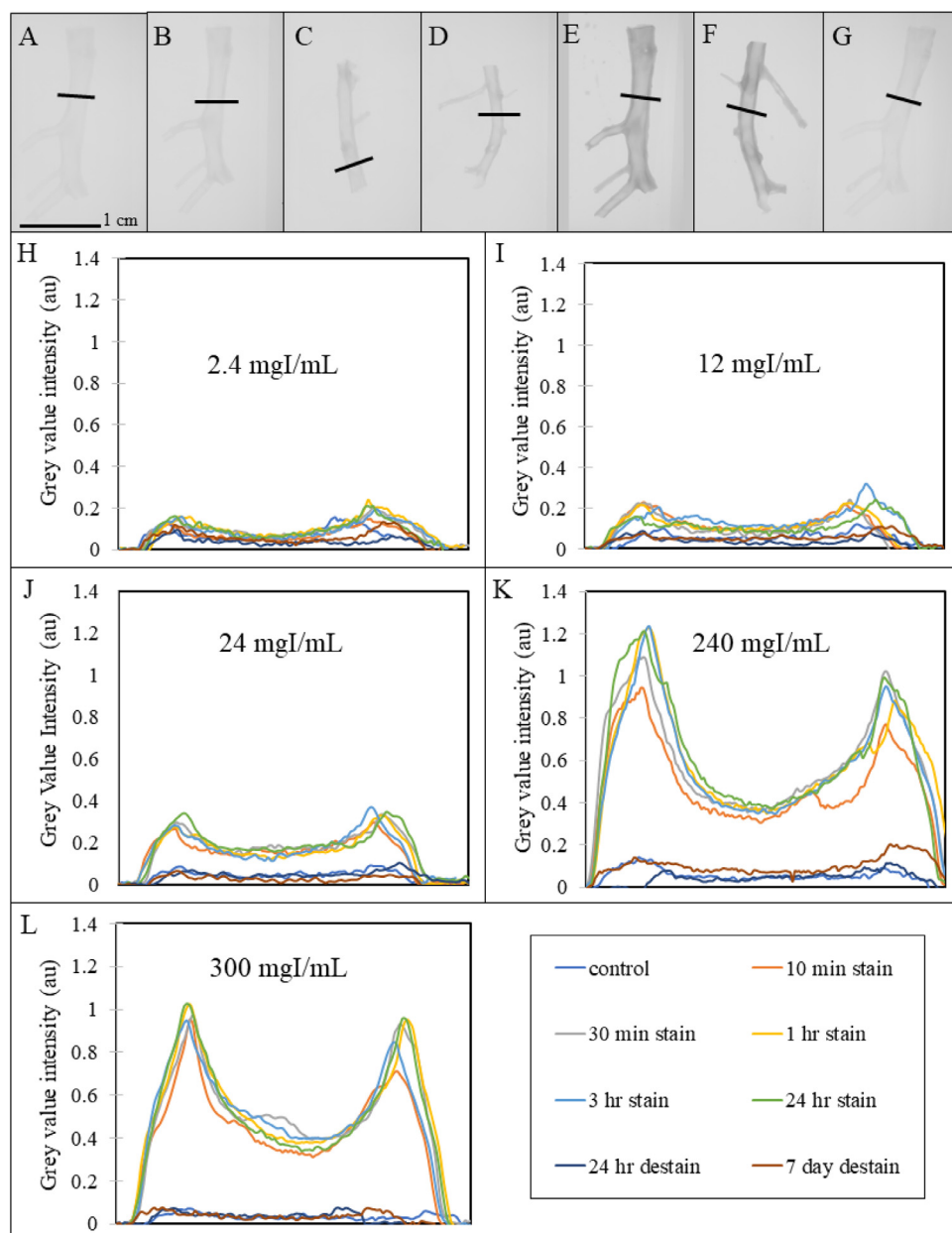


Fig. 2. Preliminary testing results. Panels A-G represent DR imaging of atherosclerotic artery segments with no stain (A), stained for 1 hr (B-F; 2.4 mgI/mL, 12 mgI/mL, 24 mgI/mL, 240 mgI/mL, and 300 mgI/mL, respectively), followed by 24-h destained in 10% neutral buffered formalin (G, previously incubated in 240 mgI/mL for 24 h). DR images of alternative incubation times are not shown. The black lines on panels A-G are representative of the location at which the line profile was generated. Panels H-L illustrate radiopacity augmentation for various concentrations and incubation times via grey value intensity profiles generated from DR images. Background grey value is subtracted from each line profile.

Table 1

DR acquisition settings. The values provided represent the system settings used to acquire all DR images for preliminary testing.

DR Acquisition Technique	
Voltage (kV)	60
Current (micro-amperes)	500
Focal Spot (microns)	20
Tube to detector (mm)	1274.78
Tube to object (mm)	213.779
Calculated Ug (mm)	0.09926
Frames Averaged	20
Acquisition time (sec)	21

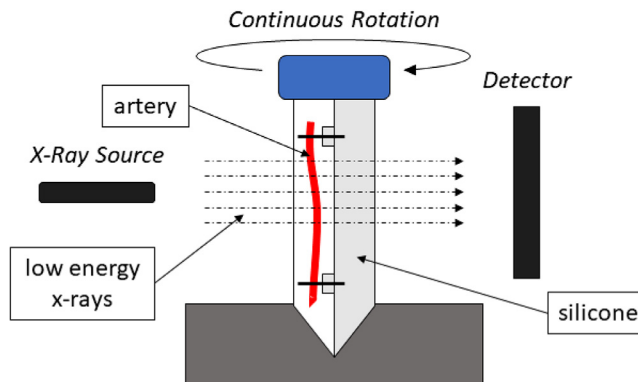


Fig. 3. Schematic of the sample mounting apparatus used for micro-CT image acquisition. Vessel segments were loaded as shown above with the proximal and distal ends pinned to silicone blocks and then the silicone base for stabilization.

4 in x 1 in were taped together at a 90-degree angle to form a stand (Fig 1A). One layer of parafilm was wrapped around the stand and the sample was placed in the centre. A second layer of parafilm was first stretched then positioned over the sample with only enough pressure to hold the sample in place (Fig 1B). Lastly, the ends of the second layer of parafilm were then adhered to the first layer of parafilm via pressure, on the back side of the positioning foam. Note that if the parafilm is folded and not uniform in thickness and is adhered to the positioning foam directly behind the sample (i.e., in the imaging path), artefact may result. Prior to staining, a direct radiograph (DR), or x-ray, was taken of the sample as a control using a North Star Imaging (NSI) X50 micro-CT machine (Rogers, Minnesota). To compare contrast enhancement and destaining, DR images were acquired after each incubation period. Following 24-h staining and DR image acquisition, samples were placed back into 10% NBF to de-stain. DR images were taken after 24 hs and 7 days of de-staining. All DR images were grouped by iohexol concentration (single samples), and line profiles were generated to determine the optimal staining concentration and incubation time combination, as well as the effectiveness of de-staining. All DR images were acquired at the parameters shown in Table 1. A summary of the preliminary testing is illustrated in Fig. 2. The data collected during preliminary testing demonstrate the augmented radiopacity of soft tissue by iohexol staining (Fig. 2). Using our acquisition settings for DR imaging, discernment of atherosclerotic plaques within the artery segments was not achieved. Atherosclerotic plaque imaging was subsequently achieved using micro-CT imaging paired with iohexol, using the optimized concentration and incubation time provided by the preliminary DR tests.

Sample Staining: The described staining protocol was then applied to samples fixed in 10% NBF, for subsequent micro-CT imaging. Coronary arteries were removed from 10% NBF and placed in 5 mL of 240 mgI/mL iohexol solution to stain for 1 h. Prior to full vessel submersion into iohexol solution, an

Table 2

Micro-CT acquisition settings. The values provided represent the system settings used to acquire micro-CT images, paired with the iohexol staining protocol derived from preliminary testing.

Micro-CT Technique	
Voltage (kV)	60
Current (micro-amperes)	500
Focal Spot (microns)	20
Tube to detector (mm)	1274.789
Tube to object (mm)	213.778
Calculated Ug (mm)	0.09922
Projections	3000
Acquisition time (min)	50

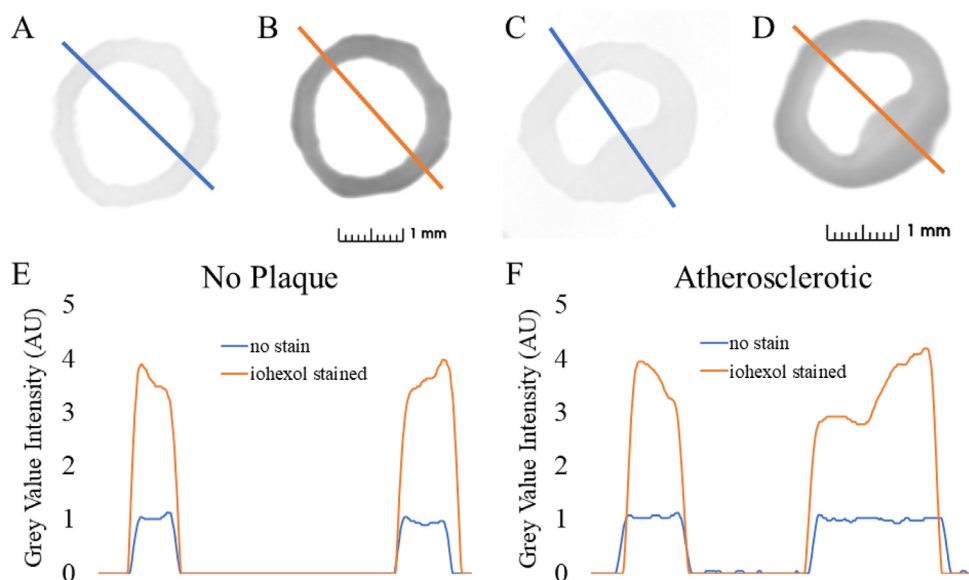


Fig. 4. Micro-CT with iohexol staining. Panels A-D illustrate micro-CT cross-sections. Panels A and B represent the same sample, free of atherosclerotic plaques, before iohexol staining (A) and after staining with iohexol for 1 hour at 240 mgI/mL (B). Panels C and D represent another sample, with atherosclerotic plaques present, before iohexol staining (C) and after staining with iohexol for 1 hour at 240 mgI/mL (D). Line profiles across the micro-CT cross-sections are illustrated by panels E and F. The blue and orange lines seen in A-D represent the approximate location at which the line profile was generated for that sample. Data points were normalized to the average plateau value of the unstained sample in each group.

intravenous catheter (Braun Medical; 20 G x 1 in) was used to gently fill the lumen with contrast agent for improved perfusion. Subsequently, the artery was removed from the contrast agent and placed on non-woven surgical gauze to remove excess iohexol solution from the vessel surface. To clear the lumen of contrast agent solution, non-woven surgical gauze was carefully placed on the proximal and distal ends of the artery section.

Micro-CT Scan Prep: The vessel section was mounted in the micro-CT imaging apparatus as illustrated in Fig. 3. A 50 mL polypropylene centrifuge tube was prepared with 25 mL of silicone (Sylgard 184, silicone elastomer) set axially in the vial. Silicone blocks approximately 1cm x 1cm x 1cm silicone (Sylgard 184, silicone elastomer) were cut to serve as spacers. The proximal and distal ends of the vessel segment were each pinned to silicone spacers using stainless steel pins, those blocks were then pinned to the silicone base set in the centrifuge tube. This setup allowed

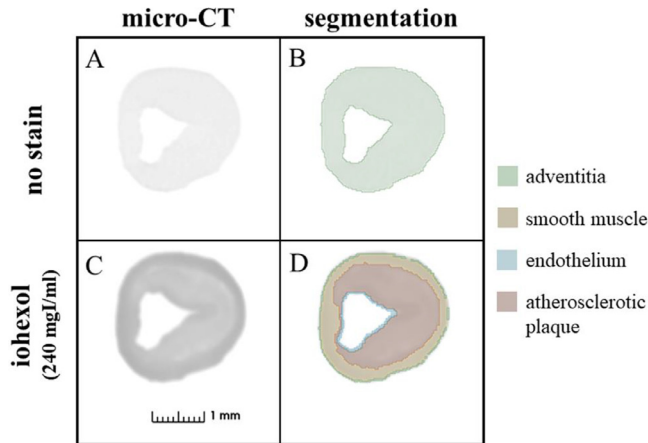


Fig. 5. 3D segmentation improved with iohexol staining. Panels A-D illustrate micro-CT cross-sections at the same location within the same atherosclerotic sample, before iohexol staining with subsequent segmentation (A and B, respectively), and following iohexol staining (240 mgI/ml for 1 hour) with subsequent segmentation (C and D, respectively).

for a section of the vessel to be suspended in air during the scan. In previous imaging attempts, we placed the vessel in fluid and gauze but reconstruction and segmentation of the vessel were difficult with these substances surrounding the vessel. Therefore, we developed the silicone block spacers. To stabilize and hold the vial upright, the centrifuge tube was then placed into a foam stand. This mounting apparatus provided a relatively low, homogeneous density surrounding the vessel, which yielded the best segmentation results. The sample was then imaged using a North Star Imaging (NSI) X50 micro-CT machine (Rogers, Minnesota).

Imaging: Vessels were scanned, using micro-CT, at the parameters defined in [Table 2](#). Scan duration for each sample was approximately 50 min. The voltage and amperage of the system were optimized for increased contrast and remained constant for all samples. Sample dehydration and sample shifting are both concerns for micro-CT imaging of coronary arteries. For the present study, sample dehydration was addressed by minimizing the image acquisition time. Sample shifting was minimal due to the security of the silicone mounting apparatus, and shifting that did occur, was corrected via NSI eFX-CT software (Rogers, Minnesota). Although sample dehydration can cause sample shifting, our definition of sample shifting is unwanted, linear movement of the sample due to failure of the silicone mounting apparatus.

Post-Imaging: Following micro-CT imaging, the vessel was removed from the micro-CT machine and returned to 10% NBF. Three-dimensional reconstruction was performed using NSI efx-CT software (Rogers, Minnesota) and output as DICOM files. These files were further analysed by a variety of post imaging software programs, such as 3D Slicer, ImageJ, or OsiriX. Results from micro-CT imaging, paired with iohexol staining, are summarized in [Fig. 4](#). Augmented radiopacity of the soft tissues comprising the vessel wall was observed with iohexol staining. [Fig. 4A-D](#) illustrate micro-CT cross-sections of two separate samples, one without plaque ([Fig 4A,B](#)) and one with atherosclerotic plaques ([Fig 4C-D](#)). In the absence of iohexol, soft tissue delineation was not achieved ([Fig 4A](#) and [C](#)). Following iohexol staining, soft tissue delineation of the vessel wall was realized ([Fig 4B](#) and [D](#)) due to augmented radiopacity within the tissues. Line profiles characterizing the grey value intensity changes are shown in [Fig 4E,F](#). Both the signal intensity and the shape of the curve were altered by iohexol staining. The transition through soft tissue layers was better resolved with a plateau region across the plaque ([Fig 4F](#)), allowing for improved segmentation during micro-CT image analysis, as evident in [Fig. 5](#). Three-Dimensional segmentation was improved due to preferential accumulation of iohexol in lipid-rich tissues ([Fig 5C](#) and [D](#)) compared with that observed in the absence of iohexol ([Fig 5A,B](#)). Removal of solution from the sample lumen improved segmentation capabilities as shown

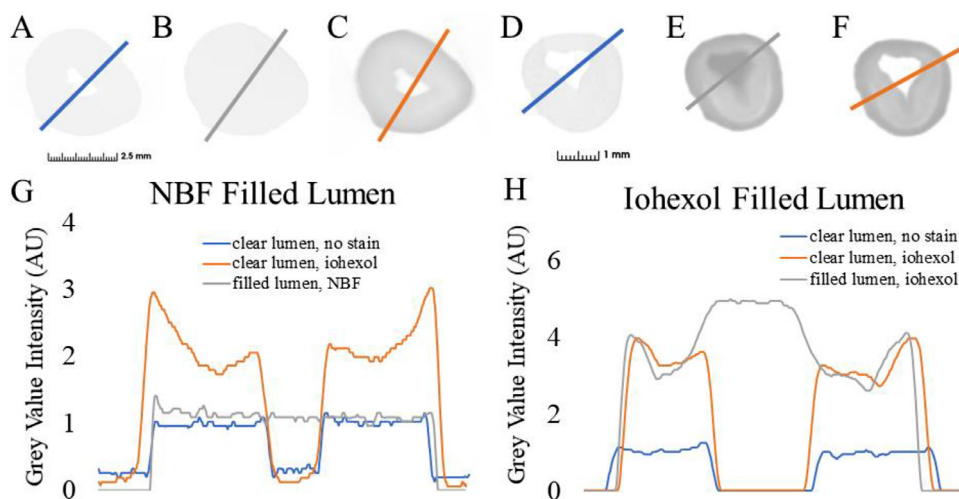


Fig. 6. Micro-CT: Solution present in lumen. Panels A-F are micro-CT cross-sections of atherosclerotic coronary artery segments. Before iohexol staining (A and D), 10% neutral buffered formalin remained in the sample lumen during scanning (B), iohexol solution remained in the sample lumen during scanning (E), and iohexol staining with a cleared lumen (C and F). Panels G and H illustrate grey value intensity plots along the orange, grey, and blue lines seen in panels A-F.

in Fig. 6. Importantly, NBF appeared to have a comparable density to the unstained tissue resulting in the inability to discern between the two volumes and therefore impaired segmentation (Fig. 6B and G). Remaining iohexol demonstrated a higher density than the surrounding tissue, allowing for segmentation to be performed (Fig. 6E and H). However, iohexol staining with the sample lumen cleared of solution yielded the best segmentation results (Fig. 6F and H). More detailed results from the micro-CT studies are reported in the co-submission paper [1].

Supplementary material and/or additional information

Histological analysis is the gold standard for analysing atherosclerotic plaques in coronary arteries. Although histologic techniques are a powerful approach to the interrogation of the composition and size of these lesions, the morphological data of plaque progression within the artery is altered or lost with these methodologies due to processing requirements and physical disruption of the tissue. Micro-computed tomography (micro-CT) imaging bridges the gap between *in vivo* and *ex vivo* studies of arterial plaques by providing a method through which *in situ* architectural characterization of atherosclerotic plaques can be collected postmortem, prior to histological sectioning.

Although there are drawbacks to micro-CT imaging, including cost and imaging artifacts, the largest barrier to implementation is the lack of inherent contrast within soft tissues. This problem has been addressed in previous studies using a variety of radiopaque contrast stains and imaging procedures [2–6]. However, these methods have not addressed imaging of atherosclerotic coronary vasculature. In addition, staining agents that have been evaluated are often irreversible, destructive to the tissue, require lengthy staining times and may result in tissue artifacts during subsequent histological analysis [7–9].

The following method applies iohexol (Omnipaque; 240 mgI/mL; GE Healthcare) for contrast enhancement of soft tissue and atherosclerotic plaques for micro-CT imaging. This agent is iodine-based, non-ionic, and water soluble, making it optimal for improved image contrast and de-staining tissue samples following image acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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