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Multiphoton Imaging of Collagen, Elastin and Calcification in Intact Soft Tissue Samples

Piyusha S. Gade¹, Anne M. Robertson^{1,2}, and Chih-Yuan Chuang²

¹Department of Bioengineering, University of Pittsburgh Pittsburgh, PA

²Department of Mechanical Engineering and Materials Science, University of Pittsburgh Pittsburgh, PA

Abstract

Multiphoton induced second harmonic generation and two photon excitation enables imaging of collagen and elastin fibers at micron level resolution to depths of hundreds of microns, without exogenous stains. These attributes can be leveraged for quantitative analysis of the 3D architecture of collagen and elastin fibers within intact, soft tissue specimens such as the artery and bladder wall. This architecture influences the function of intramural cells and also plays a primary role in determining tissue passive mechanical properties. Calcification deposition in soft tissues is a highly prevalent pathology in both older and diseased populations that can alter tissue properties. In this unit, we provide a protocol for simultaneous multiphoton microscopy (MPM) imaging and analysis of the 3D collagen and elastin structure with calcification, effective for fixed and fresh intact samples. We also provide an associated micro-CT protocol to identify regions of interest in the samples to target the MPM imaging.

Keywords

Multiphoton microscopy; collagen; elastin; calcification; extracellular matrix; micro-CT

Introduction

Soft biological tissues, such as arteries, heart, bladder and skin, are layered composites containing collagen and elastin fibers. These load bearing proteins represent the bulk of the extracellular matrix (ECM) and play a primary role in determining the passive mechanical properties of soft tissues. It is widely acknowledged that cellular phenotype and function are influenced by the properties of this fibrillar architecture. This phenotype in turn influences rates of deposition and removal of collagen fibers (Lacolley et al., 2017; Steucke et al., 2016; J. Wu et al., 2016). Hence, understanding how pathological changes to the ECM alter the stiffness and failure properties of soft tissues is crucial for advancing the treatment of diseases such as cerebral aneurysms, aortic dissection, hypertension and stroke (Cheng et al., 2018; Robertson et al., 2015). A quantitative assessment of these fibers is also important for

Corresponding author: Anne M. Robertson, rbertson@pitt.edu.

Conflict of Interest Statement

The authors do not have any conflict of interest.

the design of tissue engineered soft tissues including blood vessels and other organs (K. Lee et al., 2018; W. Wu et al., 2012). The organization of ECM proteins varies across the wall thickness and generally contains fibers oriented along multiple orthogonal planes. Therefore, there is a great need for effective 3D non-invasive imaging of the fibrous structure of intact soft tissue samples, as 2D histological studies cannot adequately capture this organization.

Multiphoton laser scanning microscopy (MPM) is a powerful technique that can be leveraged for non-invasive 3D imaging of intact tissue volumes. MPM induced intrinsic second harmonic generation (SHG) from ECM proteins like fibrillar collagen and two photon emission (2PE) of elastin, enables high fidelity imaging without the need for exogenous staining. This approach offers an important advantage over other imaging modalities by enabling simultaneous imaging of the 3D microstructure of both collagen and elastin fibers at relatively large depths (typically hundreds of microns), including the layer-specific inter-relationship of these components. The imaging depth depends on scattering and absorption coefficients of the tissue, efficiency of fluorophore and the throughput of optics. Background material on the principals of MPM imaging of biological tissues can be found in Xie et al. (Xie et al., 2012), Padmanabhan et al. (Padmanabhan et al., 2010), Dunn et al. (Dunn et al., 2006) and König (König, 2000).

While SHG and autofluorescence imaging offers multiple advantages for collagen and elastin fibers, there is a need for complementary protocols for simultaneous imaging of additional wall components using stains and fluorescent probes. Ideally, these protocols would also be effective in fresh samples. Here, we provide such a protocol for simultaneous MPM imaging of calcification along with collagen and elastin fibers that is suitable for both fixed and fresh intact tissue samples. While calcification is a pathology of numerous soft tissue diseases, particular attention has been given to arterial calcification due to its importance as a major predictor of cardiovascular events (New et al., 2011). Calcification can form in the intima of arteries through a slow, inflammation driven, atherosclerotic type process during plaque formation. A second mechanism for calcification is a non-atherosclerotic phosphate-dependent mineralization in the media that is typically associated with elastin fibers and diseased pathologies like diabetes mellitus and chronic kidney disease. Due to the involvement of the calcification with the ECM, it is important to understand the location of calcification within the wall as well its physical relationship with collagen and elastin fibers (Miller, 2016).

Since MPM imaging of intact sample volumes is a time-consuming process, it is valuable to identify regions of interest (ROI) for targeted scanning. In the first protocol in this article, we provide a methodology to achieve this objective using microcomputed tomography (micro-CT). A 3D geometric model of the sample is created along with wall thickness and calcification maps. We demonstrate how these maps can be used to guide the MPM studies. Targeted approaches of this kind are particularly important for unfixed (fresh) tissue samples which degrade over time. The second protocol provides the methodology for MPM imaging of collagen, elastin and calcification within the ROI's of the intact specimen. Fiducial markers on the tissue specimen, visible under micro-CT, are then used to identify the ROI in the sample under MPM. This protocol also provides methods to obtain quantitative data

from MPM images including fiber orientation and waviness, important metrics critical for understanding mechanical properties of soft tissues.

BASIC PROTOCOL 1 Use of Micro-computed Tomography for *A Priori* Selection of Imaging Location

To MPM scan a square millimeter of tissue (four 500µm square regions) of approximately 200µm thickness requires on the order of 1–2 hours. It is therefore valuable to identify ROI's prior to MPM imaging, particularly for fresh sample analysis. In this first protocol, non-invasive 3D micro-CT scanning of the tissue specimen is performed to generate a 3D geometric model of the sample with a corresponding wall thickness map and 3D calcification map. Regions for focused MPM analysis are then chosen using the 3D geometric model based on features such as focal areas of calcification. The preferred scan region is located on the fresh tissue sample through the use of fiducial markers that are visible in the micro-CT model. This protocol is designed for fresh and fixed tissue samples.

Materials

- Tissue sample to be imaged
- Wet gauze, Kimwipes
- Tissue dyes (Davidson Marking System, Bradley Products Inc., MN, USA)
- Dissection Scope (Olympus SZX10, Tokyo, Japan)
- 200 – 300 µm polystyrene beads (Polysciences Inc., Warrington, PA)
- Glue (Loctite 404, McMaster-Carr, IL, USA)
- 1.5ml clear microcentrifuge tube (Thermo Fisher Scientific, PA, USA)
- Mounting brass stub
- Desktop Microcomputed Tomography (Micro-CT) scanner (Skyscan 1272, Bruker Corporation, Belgium, resolution = 0.35 µm (16Mp) at highest magnification)
- Computer hardware and software for image acquisition, reconstruction (NRecon, Bruker Corporation, Belgium), and analysis (Simpleware ScanIP, Synopsys, Mountain View, CA, USA)

Protocol Steps

Application of Fiducial Markers (dye and beads)

1. Dry the sample using Kimwipes until no excess fluid is seen on the tissue surface.
2. Place the sample under the dissection scope and mark fine dots in a grid structure using the tissue dyes (Figure 1A). This grid structure is visible under MPM analysis and enables mapping of MPM imaging locations to CT images, details of which are provided in the next step. The dye requires approximately 5

minutes to bond to the tissue surface. It is advisable to turn off the dissection scope light during this time to avoid any potential damage to the tissue.

While in principal any color of the Davidson dyes could be chosen for tissue marking, the chemical makeup of each dye is different.

Therefore, in addition to having different contrast with the tissue, their affinity with the tissue also varies. We found blue and red dyes provide the best results for vascular tissues. The yellow and orange dyes do not provide sufficient contrast with the tissue surface color. Green and black dyes are highly miscible in water and can lead to excessive spreading on the tissue surface.

3. Attach marking beads over the dye location using a small amount of glue (Figure 1A).

Loctite 404 glue was chosen for attaching the beads as the application of this adhesive is easily controlled and little glue volume is needed. These features ensure there are negligible changes to tissue material properties. The beads are visible under micro-CT; however, they can detach and separate from the tissue during the preparations for MPM scanning. Although the dye mark cannot be detected under micro-CT, it is more permanent. Therefore, this bead/dye combination guarantees that beads will be visible under micro-CT and dye marks (and possibly some beads) will be visible under the MPM without excessive use of adhesive. This ensures the presence of sufficient markers to register points from the surface of the 3D micro-CT data set to MPM images.

Tissue Mounting in Micro-CT Scanner

4. Place wet gauze or Kimwipes at the bottom of a 1.5 ml clear microcentrifuge tube to prevent sample dehydration during scanning. Then place the sample vertically on top of the gauze at the center of the tube (or on top of an added piece of Styrofoam to help position sample) and seal it tightly (Figure 1B).

To ensure maximum field of view, place the sample in the center of tube, symmetrically located with respect to the vertical axis.

Alternatively, if the sample is very small, cling wrap (Glad®, CA, USA) can be used to mount the sample on the stub (Barbe, 2014). This particular brand of wrap was found to avoid substantial x-ray attenuation.

5. Place the microcentrifuge tube vertically on the mounting brass stub and seal the tube to the stub using parafilm sealing film (Figure 1B).
6. Mount the stub onto the micro-CT scanner.

Tissue Scanning

7. Scan the sample using the settings outlined here while maintaining a scan time of less than 2 hours for fixed, and less than 1.5 hours for fresh sample to prevent

sample dehydration. Note that the settings will vary slightly depending on the sample dimension. Samples that have dimensions less than $1\text{ cm} \times 0.5\text{ cm}$ can be scanned at 50kV, 200 μA with an image pixel size of $1 - 3\mu\text{m}$, frame averaging of 8–10, rotation step size of $0.1 - 0.4$ degrees, scanned 180 degrees around the vertical axis. Since this protocol pertains to biologic tissues, no filter is used for scanning. However, aluminum, copper or a combination of the two can be used for samples with higher density.

A flat field correction is done prior to every scan to ensure optimal image contrast.

Two hours is a conservative estimate for micro-CT imaging of fixed tissue samples using this procedure. Thicker samples will dry out more slowly. However, if the sample does dry out, the corresponding change in sample volume will generate misalignment artifacts that will be seen during post-processing.

Creation of Sample Volume and Selection of Region of Interest (ROI)

8. In order to ensure optimal image quality, correct the images for the most commonly occurring CT artifacts like beam hardening and ring artifact. Then, select an ROI and corresponding volume of interest for reconstructing raw CT images. The interested reader can reference the NRecon User Manual for further details on image reconstruction.
9. Segment calcification from the reconstructed tissue data set using one of a variety of image processing software (e.g. MATLAB, ITK, VTK, Slicer 3D etc.). In this protocol, we use Simpleware ScanIP for image analysis owing to the ease with which high quality surface mesh from CT data can be generated. Segment the calcified regions (regions of relatively high grayscale value) using a combination of thresholding, connected regions filtering, Boolean operations and Gaussian smoothing to create a calcification mask. Create a second mask for the tissue with beads. To enable 3D visualization of the calcification within the tissue sample, overlay these masks (Figure 1C).

The interested reader is referred to the supplementary material section for detailed methodology for segmentation and analysis of calcification and tissue.

10. Select the ROI for MPM analysis using the overlaid image based on the scientific objective of the investigation. For example, in Figure 1C, a ROI is selected for targeted MPM study (rectangle) due to the clustering of calcification in that region.
11. MPM imaging can also be targeted based on geometric features such as sample thickness or interfaces between thick and thin regions. These geometric features can be evaluated from the surface/volume mesh for the sample. In this protocol, Geomagic (3D Systems, NC, USA) was used for this purpose using an analysis function for wall thickness maps (Figure 1D).

BASIC PROTOCOL 2 Simultaneous Second Harmonic Generation and Two Photon Excitation Imaging of Collagen, Elastin and Calcification

In this protocol, we describe how to use MPM induced SHG and 2PE imaging to detect collagen and elastin in soft tissue along with calcification, made visible using a far infrared tracer. Using this technique, it is possible to non-destructively understand the three-dimensional structural relationship between ECM and calcification. This protocol can be used to image fresh or fixed tissue samples. We illustrate some key results using a fresh human cerebral artery and a fixed specimen from a human cerebral aneurysm.

Materials

- Tissue sample to be imaged
- 10× Phosphate buffered saline (PBS)
- Near infrared bisphosphonate-based calcium tracer - Osteosense 680 (Perkin Elmer, MA, USA) (See Reagents and Solutions)
- 4% paraformaldehyde (PFA)
- Custom made sample chamber (described in Figure 2) assembled from two threaded metallic rings (for example, IDEX health and Science, NY, USA), a silicone gasket (for example, Roettele Industries Inc., CA, USA) and two 18 mm diameter round microscope glass coverslips (Thermo Fisher Scientific, PA, USA). Thickness of gasket determines internal chamber height which can be changed depending on sample thickness.
- Olympus Multiphoton Microscope (FV 1000, Tokyo, Japan)
- Spectra-Physics DeepSee Mai Tai Ti – Sapphire laser (Newport, Mountain View, CA)
- 1.12NA 259 MPE water immersion objective
- Computer hardware and software for 3D reconstruction of MPM images and fiber analysis, for example, Imaris 9.2 (Bitplane, CT, USA) or CT-Fire, an open source software developed by researchers at the University of Wisconsin Madison (Bredfeldt et al., 2018)).

Protocol Steps

Sample preparation with calcium stain

1. Dilute the calcium tracer solution 1:50 from the original stock.
2. If staining a fresh sample, incubate the intact sample in the calcium tracer for 24h at 4°C in dark.
3. If staining a fixed sample, fix the intact sample for 24h in 4% PFA. Rinse the sample and incubate it with the calcium tracer for 24h at 4°C in dark.
4. After incubation, rinse the sample thoroughly with 10× PBS.

5. Mark the tissue as outlined in the Basic Protocol 1.

Sample mounting

6. It is important that the sample is securely held during MPM imaging to minimize artifacts from sample movement. As the soft tissue is often composed of layers, it can be advantageous to slightly flatten the tissue so that it can be imaged orthogonal to these layers. For these purposes, we used a simple custom-made chamber that can be placed on the MPM stage (Figure 2). The chamber consists of two concentric metallic rings, having threads on the inner and outer surface respectively, two round coverslips and a gasket (Figure 2A). To assemble, place a round glass coverslip within one ring, place the gasket on the coverslip, followed by the tissue sample. Fill with 10× PBS and place the second cover slip over the tissue with metallic ring on top (Figure 2B, C). Ensure that no air bubbles are present prior to tightening the coverslip-ring assembly.

Once assembled, notation can be marked on the outer coverslip to identify the imaging side (e.g. luminal) and, if desired, a grid can be drawn on the glass to clarify the orientation (Figure 3A). Spacing between the rings can be altered by selecting gaskets of different thicknesses.

As an alternative, hanging drop slides (Fisher Scientific) of appropriate depth can be used to secure the sample. These are available in varying depths from 1.4 – 3.2 mm. Place the sample in the slide, fill with PBS and cover with disposable coverslips. The sample will generally be held more securely and flattened more effectively with the custom chamber.

Image acquisition

7. Place custom chamber on the microscope stage and add water on the top cover slip of the chamber so the lens is immersed in water during imaging.
8. Select the appropriate settings for imaging. The following settings work well for imaging collagen, elastin and calcification with a 1.12NA 259 MPE water immersion lens and 25× objective: 800nm excitation wavelength, dwell time of 8μs/pixel, a scan pixel count of 1024 × 1024 and laser intensity of 7%.

Further details on selection of MPM settings can be found in Xie et al. (Xie, 2012).

9. Take sequential scans across the thickness (Z-stack series) at 2 μm intervals. The location for the first and last images of the Z-stack series are chosen based on a fast pre-scan using a coarse setting.

Samples can be scanned from both the luminal and abluminal sides and can typically be imaged to a depth of 200 – 300 μm without optical clearing, depending on the signal intensity from the components of the ECM. For example, the elastin in the IEL of cerebral arteries has strong autofluorescence that can prevent imaging through to the medial

collagen fibers for unloaded samples. Although, when the cerebral artery is mechanically stretched, the medial collagen can usually be imaged from the luminal side.

10. Collect SHG signal from collagen using backscatter epi detectors and 350 – 450 nm Chroma emission filters with a 50 spectral bin (Brattleboro, VT, USA). Collect the intrinsic elastin 2PE signal using a 500 – 550 nm filter and calcification using the fluorescent calcium tracer signal in the 665 – 735 nm range for both fixed (Figure 3C, D) and fresh samples (Figure 4).

The emission range for the calcium tracer provided by the manufacturer was used to select the optimal filter to use for MPM scanning. An excitation wavelength of 800 nm provided good signal quality for the calcium tracer, thereby allowing simultaneous imaging of collagen, elastin and calcification. As a result, it is possible to see all three components in images and 3D reconstructions where the signals are combined, enabling analysis of the interaction between these three components.

Three-dimensional image reconstruction

11. Create 3D image volumes from the acquired Z-stack series of images using software such as Imaris 9.2 (Bitplane, CT, USA) (Figure 3,4).

Quantitative Analysis of Collagen and Elastin Fibers—Collagen, elastin and calcification can be quantitatively analyzed in the individual MPM images, or in the 3D reconstructed stacks as described here.

Collagen fiber orientation and waviness

12. Calculate metrics of fiber orientation and waviness on each individual axial slice or by tracing collagen fibers using manual filament tracing (Figure 5A). In this protocol we used the commercial software Imaris 9.2 (Bitplane, CT, USA) for manually tracing fibers. Ensure that the filament captures the curves of the fibers to obtain accurate measurements of fiber waviness (Hill et al., 2012). Waviness and fiber orientation can be determined using built-in functions within Imaris. The variation of these metrics across the wall thickness can be visualized using, for example, a “heat map” or rose-plot (Figure 5B, C).

Collagen fiber orientation and waviness are important structural features of soft tissues that impact their mechanical function. For example, fiber waviness enables the sample to undergo large deformation with very little load (high compliance) as seen in arterial collagen fibers. Once the collagen fibers are straightened, they contribute to load bearing, leading to a stiffer response at higher strains (Hill, 2012). By obtaining quantitative descriptions of these features, tissue structure can be compared, for example, between treated and untreated tissue or between diseased and healthy tissue. Furthermore,

this information can be used to develop mathematical equations (constitutive models) with which to model the mechanical response of these tissues (Hill, 2012).

The conformation of the collagen and elastin fibers can be extracted from single planar images either from manual tracing described here, or from automated methods that include 2D discrete Fourier Transform analysis (Bayan et al., 2009), and a combination of fast Fourier Transform and wedge filtering (Schriebl et al., 2012). We find the fiber tracing preferable for evaluating the fiber straightness (inverse of waviness) and obtaining average fiber orientation for wavy fibers.

Collagen and calcification areal density

13. Calculate calcification and collagen areal density by analyzing the separate color channels in each image. Using a custom-written MATLAB script (MATLAB 2017A, MA, USA) (*provided in online supplementary material*), load images and separate color channels. Segment each of the constituents (collagen and calcification) using Otsu segmentation in their respective channels. Calculate the area of segmented pixels in each slice and sum up the segmented area across the volume to calculate total areal density in the image volume (Figure 5D).

REAGENTS AND SOLUTIONS

Osteosense stock solution preparation

- a. Add 1.2 mL of 1× PBS to the reagent vial and gently shake or vortex to ascertain the agent is in solution.
- b. Keep the stock solution wrapped in aluminum foil at 4°C for < 2 months.

COMMENTARY

Background Information

Quantitative evaluation of the microstructure of biological tissue samples is essential for understanding tissue function, disease pathologies, disease progression and the role of therapeutic agents. Classical histological techniques have been instrumental in elucidating the microstructural anatomy of various tissues which has improved understanding of the related physiology and also contributed to the design of novel treatment strategies for diseases. However, these studies are conducted on small, thin (<10µm) tissue sections thereby capturing a small subset of the tissue sample in a single 2D plane, impeding efforts to understand the 3D structure of the tissue. Historically, 3D visualization was achieved through 3D reconstruction of serial sections (Lauderdale et al., 1972). Furthermore, recent advances such as automated serial sectioning have increased sectioning rates (Spowart, 2006). The development of image alignment (Szeliski, 2006), montaging (Fiala, 2005), and auto-focusing have markedly increased the utility of serial sectioning for 3D visualization of tissue microstructure (O'Connell et al., 2009). However, histological techniques are inherently destructive, have to be conducted on fixed, processed samples and are associated

with a number of artifacts including tissue shrinkage, change in morphology, tears, detachment of layers, folds and can be applied to only small volumes of tissue (Rastogi et al., 2013). Therefore, there is a great motivation to develop protocols for directly imaging whole volume samples.

MPM leverages the intrinsic SHG and 2PE signal from collagen and elastin, respectively to provide high imaging depth, typically on the order of hundreds of microns. This is in contrast to confocal imaging which has significantly lower imaging depth ($< 100\mu\text{m}$) due to the lack of localization of imaging to a single spot, increasing out-of-plane emission. However, many tissues, such as the walls of many extracerebral vessels in humans and larger mammals, are thicker than 1 mm and therefore beyond the MPM imaging depth using the approaches described here. Both confocal and MPM imaging depth can be increased by optically clearing the tissue using solutions with refractive indices closer to those of the wall components, reducing light scattering (Muntifering et al., 2018). Although this process cannot be performed on fresh tissue samples, it can increase imaging depths in fixed samples by orders of magnitude.

Imaging of intact, fresh specimens using MPM also opens up the possibility of analyzing changes in tissue structure during mechanical loading. For example, using custom-MPM compatible uniaxial and biaxial mechanical testing devices, our group has elucidated the role of collagen fiber waviness in determining the material properties for rabbit carotid artery (Hill, 2012) and rat urinary bladder wall (Cheng, 2018), respectively. Thus, MPM is crucial not only for understanding the anatomical microstructure, but also for developing mathematical descriptions of the role of this microstructure in determining the mechanical function of the tissue. These models can be used to quantitatively compare tissue in health and disease as well as to test hypothesis about the mechanical role of wall components.

Intrinsic MPM-induced SHG and 2PE signals can be combined with fluorescent tracers that do not interfere with the emission range of collagen and elastin to simultaneously image other wall components such as cell nuclei, flavins, NAD(P)H, porphyrins and lipofuscins. A list of stains with their emission spectra is provided by Bestvater et al. (Bestvater et al., 2002). In this protocol we introduce the use of a calcium tracer, Osteosense 680 (Perkin Elmer, MA, USA) for use with MPM. This tracer was previously used with confocal microscopy by Hutcheson et al. (Hutcheson et al., 2016) to study the role of microcalcification in atherosclerotic plaque rupture using collagen fiber SHG signal. Here, we adapted their protocol for MPM analysis of both fresh and fixed samples, enabling simultaneous imaging of calcification and intrinsically fluorescing ECM components (collagen, elastin) up to depths of hundreds of microns. *In vivo* two photon calcium imaging has been extensively used in the neuroscience community to detect neuronal activity at high depth and high resolution (Cheetham, 2018; Mittmann et al., 2011; Stosiek et al., 2003). In this protocol, the focus on complimentary calcium staining is motivated by its role in soft tissue pathologies. For example, ectopic calcification of vascular tissue is associated with cardiovascular risk factors such as hypertension, inflammation and chronological aging (Durham et al., 2018; Lakatta, 2016). While it has long been understood that large calcification deposits will alter the mechanical and transport properties in arteries, the potential role of even small (micro) calcifications in altering tissue mechanics is being

explored in the context of atherosclerotic plaque rupture (Hutcheson, 2016; Vengrenyuk et al., 2006). This unit provides the techniques necessary for achieving the resolutions required to image the calcification-ECM interaction and ultimately can be used to quantify the effect of calcification on ECM recruitment and mechanical function during loading.

Micro-CT, conventionally used for imaging of hard calcified tissue inclusions, has also been used in imaging soft non-calcified tissue, especially in the presence of x-ray contrast agents. Metscher (Metscher, 2009) provides a comprehensive overview of various contrast agents suitable for soft-tissue x-ray imaging. While the micro-CT protocol described here was introduced for the purpose of selecting regions of interest within the tissue sample, it can also be used for a variety of applications that require structural assessment. For example, our group has previously demonstrated the use of high resolution micro-CT to analyze the role of fabrication technique on tissue engineered vascular grafts (S. H. Lee et al., 2017) as well as their *in vivo* and *in vitro* degradation rates (Gade et al., 2017).

The combination of non-destructive imaging modalities such as micro-CT and MPM offers unique opportunities for targeted assessment of the wall content in heterogeneous materials. The resolutions achieved by both these techniques for imaging calcification are comparable to that of standard histological techniques, while avoiding common artifacts associated with mechanical sectioning and staining of samples. The protocol introduced here uses micro-CT to first image the entire samples to guide the selection of ROI's for detailed study of ECM-calcification interface. Using the micro-CT model of the physical sample, the MPM microstructural data can also be mapped to computational data for blood flow and mechanical loading within the wall (Cebal et al., 2016; Cebal et al., 2018). Mapping of this kind is particularly important when there are heterogeneities in the tissue, such as those due to disease or even structural heterogeneities such as at the bifurcation of arteries.

Critical Parameters

1. Sample stability during imaging

The sample must be held stationary during MPM imaging, either using a custom chamber such as the device described here or by placing the sample in a slide with a well. Furthermore, clay (Renishaw Inc, Part No. A-1085-0016) can be used to ensure that the device itself is affixed to the micro-positioning stage during imaging and there is negligible device movement.

1. Sample stability and centering in CT

If the sample cannot be held vertically in the center of the mounting tube, place some Styrofoam (or other material of similar low densities) around the sample to ensure that it doesn't move. Image quality can also be compromised by "movement" caused by the sample drying out. Ensure that wet gauze is placed at the bottom of the tube prior to scanning. If the sample is still drying out, wet gauze can also be placed on the top of the sample.

1. Micro-CT scanning time

As outlined in Protocol 1, sample scanning time must be less than 1.5 h for fresh and less than 2h for fixed tissue samples in order to prevent sample dehydration and image

movement artifacts. This can be achieved by (a) reducing scan resolution, (b) increasing scanning rotation angle, (c) reducing frame averaging.

Troubleshooting

Anticipated Results

If the protocols outlined above are followed, simultaneous MPM images of collagen, elastin and calcification can be acquired and mapped to the 3D *in silico* model of the physical sample, created from the micro-CT data. These images can help in better understanding the ECM structure and its integrity from either qualitative and/or quantitative analysis. For example, in some cases damaged fibers will be revealed and recognized as a diseased pathology, Figure 3C, D. The data can then be used to quantify the local interaction and morphological changes to elastin and collagen due to the presence of calcification. The location of calcification within the wall can be quantified and metrics like percentage of calcified area in the sample can be calculated (Cebal et al., 2018). Namely, the micro-CT model can serve not only as a mapping tool but also provides geometric information about the sample and provides a bulk assessment of calcification burden. In addition to guiding MPM studies of the tissue, the micro-CT data can be used to select regions for mechanical testing within the large tissue samples. For example, guided by the micro-CT data, specimens for mechanical testing can be cut from the larger sample so as to include clusters of micro-calcifications. The tissue specimen will then be well suited for evaluating the impact of these particles on tissue mechanical properties. Quantitative data on collagen fiber orientation and waviness enables the development of structurally motivated constitutive models that mathematically describe the mechanical properties of the tissue sample.

Time Considerations

The time required for this protocol from the time the sample is acquired will vary based on whether the sample needs to be imaged fresh or fixed, the size of the sample and the number of locations that need to be scanned using the MPM. For fixed tissue, a fixation time of 24 h followed by 24 h of incubation with calcium tracer leads to 48 h for sample preparation prior to any imaging. The preparation time for a fresh sample is 24 h. The fixed and fresh samples can be scanned using micro-CT for less than 2h depending on sample size. The MPM scanning time is the same for both fresh and fixed samples and will vary based on the depth and area of scanning. Each $500\mu\text{m} \times 500\mu\text{m} \times 200\mu\text{m}$ section requires about 20 minutes at the settings outlined in the basic protocol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Hutcheson JD, Goettsch C, Bertazzo S, Maldonado N, Ruiz JL, Goh W, Yabusaki K, Faits T, Bouten C, Franck G, Quillard T, Libby P, Aikawa M, Weinbaum S, & Aikawa E (2016). Genesis and growth of extracellular vesicle derived microcalcification in atherosclerotic plaques. *Nature Materials*, 15(3), 335–343. doi:10.1038/NMAT4519 [PubMed: 26752654] Describes the use of calcium tracer with confocal microscopy on fixed atherosclerotic human tissue samples.

Robertson Anne M., and Watton Paul N.. “Mechanobiology of the arterial wall” *Modeling of transport in biological media*. Elsevier, New York (2013): 275–347. A book chapter that reviews information on the extracellular matrix in arteries including the role of tissue microstructure in determining tissue function.

Internet Resources

<https://loci.wisc.edu/software/ctfire> Website for downloading and obtaining documentation for the open source software program CT-Fire for automated fiber tracing.

<https://www.thermofisher.com/us/en/home/references/molecular-probes-the-handbook/technical-notes-and-product-highlights/fluorescent-probes-for-two-photon-microscopy.html> Website detailing the fluorescent probes available for multiphoton imaging.

Significance Statement

This article provides methodology for non-destructive, multiphoton microscopy (MPM) imaging of fibrillar proteins within intact soft tissues at cellular level resolution. Imaging can be performed in fresh or fixed samples to depths of hundreds of microns, with the possibility of additional use of exogenous stains for imaging complimentary structures. The first protocol explains the use of micro-CT for *a priori* selection of regions of interest to target the MPM scanning in the second protocol. The resulting data can be analyzed to obtain 3D quantitative information about the inter-relationship and conformation of these constituents that is vital for understanding soft tissue function, disease progression, and treatment efficacy. The protocol can be combined with mechanical testing to image changing extracellular matrix during loading.

Figure 1:

(A) Dissection scope image of a PFA fixed aneurysm, (B) Mounting setup for micro-CT scanning and (C) a 3D reconstructed image of the micro-CT data for an aneurysm sample with calcification (yellow) and tissue (grey). Note the beads on both the physical and reconstructed sample. These beads and dyes serve as physical markers for mapping MPM images to 3D CT images and the physical sample. (D) Wall thickness map of aneurysm sample constructed from micro-CT data. This wall thickness map can be used for selecting appropriate ROI for MPM imaging.

Figure 2:
Ring assembly for MPM imaging. (A) Two round threaded metallic rings with coverslips and appropriately sized gaskets, (B) Sample placed onto one half of the assembly and immersed in 10× PBS. (C) Completed assembly after screwing the two metallic rings together.

Figure 3:

(A) Aneurysm sample mounted in custom-made device to hold tissue sample steady and flat during the course of MPM imaging. (B) PFA fixed aneurysm sample marked with tissue dye and polystyrene beads, (C) Montage of projection of 3D stacked MPM images of collagen (red) and calcification (magenta). (D) Zoomed image of location D. Note the degraded collagen fibers in regions that are occupied by calcification. Each image is a $500\ \mu\text{m} \times 500\ \mu\text{m}$ projected stack of $2\ \mu\text{m}$ thick sections.

Figure 4:

Projections of 3D stacks from MPM imaging of a fresh native human cerebral vessel, imaged en face from lumen (top panel) and albumen (bottom panel) sides. (A) Internal elastic lamina (IEL) (green) with visible fenestrations, (B,C) Calcification (magenta) visible on the lumen side along the IEL. (D) Thick, wavy adventitial collagen fibers (red) and (E,F) calcification (magenta) adjacent to the IEL. Each image is a $500\ \mu\text{m} \times 500\ \mu\text{m}$ projected stack of $2\ \mu\text{m}$ thick sections

Figure 5: Manual fiber tracing to determine collagen fiber orientation and crimp using Imaris 9.2. (A) Volume projection of collagen fibers (images in Figure 4D) with fiber tracings. Each image is a $500\ \mu\text{m} \times 500\ \mu\text{m}$ projected stack of $2\ \mu\text{m}$ thick sections (B) Circular rose plot of collagen fiber orientation distribution (C) Heatmap of fiber straightness as seen across the imaged wall thickness of the sample. The top row represents the luminal side and bottom row represents abluminal side of sample. (D) Areal density of calcification and collagen fibers for the volume shown in Figure 4E–F.

Table 1.

Troubleshooting guide for MPM imaging of collagen, elastin and calcification.

Problem	Possible Cause	Solution
Poor or no calcification signal	Inadequate diffusion of calcium tracer due to high (>450µm) sample thickness.	Increase incubation time with calcium tracer. Check if solution is still viable (is within 2 months of preparation date).
Poor or no collagen, elastin signal and high background noise	Improper laser intensity	Optimize laser intensity (typical range = 4 – 7%)
	Improper wavelength	Optimize wavelength (typical range = 800 – 870 nm)
	Inadequate dwell time	Optimize dwell time (typical range = 4 – 8µs/pixel)
	Sample movement	Ensure the sample is fixed to stage. Fasten the imaging device to stage using putty
	Inadequate immersion of lens in water	Ensure lens is submerged in water