Imaging of cardiovascular structures using near-infrared femtosecond multiphoton laser scanning microscopy

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Abstract. Multiphoton imaging represents a novel and very promising medical diagnostic technology for the high-resolution analysis of living biological tissues. We performed multiphoton imaging to analyzed structural features of extracellular matrix (ECM) components, e.g., collagen and elastin, of vital pulmonary and aortic heart valves. High-resolution autofluorescence images of collagenous and elastic fibers were demonstrated using multifluorophore, multiphoton excitation at two different wavelengths and optical sectioning, without the requirement of embedding, fixation, or staining. Collagenous structures were selectively imaged by detection of second harmonic generation (SHG). Additionally, routine histology and electron microscopy were integrated to verify the observed results. In comparison with pulmonary tissues, aortic heart valve specimens show very similar matrix formations. The quality of the resulting three-dimensional (3-D) images enabled the differentiation between collagenous and elastic fibers. These experimental results indicate that multiphoton imaging with near-infrared (NIR) femtosecond laser pulses may prove to be a useful tool for the nondestructive monitoring and characterization of cardiovascular structures. [DOI: 10.1117/1.1896966]

Keywords: near-infrared femtosecond laser scanning microscopy; multiphoton imaging; autofluorescence; extracellular matrix; heart valve.

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1 Introduction

The differentiation of multiple elements of the extracellular matrix (ECM), which discloses the precise architecture of tissues, is of great value in gaining structural and diagnostic information. In cardiovascular structures such as heart valves, the primary ECM components are arranged in leaflet layers (arterialis, fibrosa, spongiosa, ventricularis) to withstand the considerable tensile and flexion forces.[1,2] The outer layers (ventricularis as the inflow side and arterialis/fibrosa as the outflow side) are composed of collagen and elastin. The central layer, the spongiosa, consists primarily of proteoglycans, thus allowing slippage of the two surface layers across one another as the valve flexes.[3,4] However, to understand the function of healthy and diseased valve tissues, a complete understanding of the arrangement and modification of the major structural elements, such as elastic and collagenous fibers, is crucial.

So far, visualization of collagenous and elastic fibers has required the invasive removal of tissue samples (biopsies), slicing by mechanical tools (microtomes), embedding, fixation, and histological or immunohistochemical staining procedures.[5–7] Therefore, a detection of cellular and subcellular components within their natural environment has not been possible. The near-infrared (NIR) femtosecond laser scanning microscopic technique is a significant advance in the field of optical imaging methods and might offer an attractive microscopical strategy in overcoming the current limitations.

The use of multiphoton fluorescence excitation based on the simultaneous absorption of two or more NIR photons[8] enables the unique nondestructive high-resolution deep tissue visualization of endogenous fluorophores.[9] The application of NIR 80 MHz femtosecond laser pulses to a scanning device enabled the three-dimensional (3-D) autofluorescence imaging of distinct fluorophores, such as NAD(P)H, collagen, elastin, melanin, or flavins with blue/green fluorescence.

By tight focusing of ultrashort laser pulses within the subfemtoliter focal volume of an objective of high numerical aperture the required high transient gigawatt per square centimeter light intensities can be achieved. Due to the use of laser wavelengths between 700 and 1100 nm and appropriate mean laser powers, the tissue absorption in out-of-focus regions is negligible. Moreover, significant photobleaching as well as cell and tissue damage can be avoided.[10,11] Furthermore, second harmonic generation (SHG) can be used as a second-

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order nonlinear optical process imaging tool for examining endogenous structural proteins.[12,13] SHG leads to the formation of ultraviolet (UV)/blue radiation at a wavelength of exactly half the incident wavelength. This is an absorptive process and requires laser interaction with a medium lacking a center of symmetry such as an interface, for example, a membrane, or the chiral structure collagen.[14,15]

This paper presents results obtained by multifluorophore, multiphoton excitation at two different wavelengths and optical sectioning. The objective of this work was to demonstrate high-resolution autofluorescence and SHG images of the ECM of living ovine pulmonary and aortic heart valve leaflets. The achieved results were compared to routine bright-field light microscopy and electron microscopy.

2 Methods

2.1 *Source of Material*

Ovine hearts were obtained from a local slaughterhouse and immediately transferred to the laboratory. Pulmonary and aortic valves were excised from the ovine hearts and dissected of adherent fat and most of the myocardium, leaving only a thin ridge of subvalvular muscle tissue and the artery. The valves were stored in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, California) with fetal calf serum (FCS, Invitrogen) at 4 °C for further processing.

2.2 *Histology and Immunohistochemistry*

Pulmonary and aortic valves (leaflets including artery and cardiac muscle) were rinsed in phosphate-buffered saline (1×) (PBS, Invitrogen). Specimens of each valve were frozen in liquid nitrogen. Semithin sections (5 to 8 μm) were cut by a Leica Jung CM 3000 cryostat (Leica, Vienna, Austria) and finally processed for histological staining and immunohistochemistry.

Russell-Movat-Pentachrome stain[5] was used to demonstrate extracellular matrix components such as collagen, elastin, and glycosaminoglycans (GAGs). Elastic fibers were stained by the Weigert resorcin-fuchsin staining method.[6] Immunohistostaining was carried out using standard indirect immunoperoxidase (strept)avidin-biotin techniques, as reported previously.[16] A monoclonal antibody against elastin (1:50; DPC Biermann, Bad Nauheim, Germany), a polyclonal antibody to collagen type I (1:50; DPC Biermann), a polyclonal antibody to collagen type III (1:50; DPC Biermann), and a polyclonal antibody to collagen type IV (1:50; DPC Biermann) served as primary antibodies. The (strept)avidin-biotin-complex technique was performed by using the VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, California) including a biotin-labeled secondary mouse antibody. Sections were analyzed by using routine bright-field light microscopy (Axiovert S 100, Zeiss, Jena, Germany).

2.3 *Electron Microscopy*

Directly after dissection of pulmonary and aortic heart valve leaflets, specimens were washed in 0.1 M cacodylate buffer, pH 7.2 (Fluka, Buchs AG, Buchs, Switzerland), supplemented with 5% sucrose (Serva Electrophoresis GmbH, Heidelberg, Germany), and fixed for 2 h at 4 °C in 3% glutaraldehyde (Fluka), containing 0.1 M cacodylate buffer. After fixation, the tissues were treated with 1% osmium tetroxide (OsO_4, Paesel+Lorei GmbH, Hanau, Germany) in 0.1 M cacodylate buffer for 2 h at room temperature (RT) and were further processed as previously described.[17] Ultrathin sections were cut with a Reichert-Jung Ultracut F microtome (Leica), samples were collected on fomvar-coated (Fluka) copper-rhodium grids and stained with 1% uranyl acetate (Polysciences Inc., Warington, Pennsylvania), 0.05% tannin acid (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and 2.5 mg/ml lead citrate (Polysciences Inc.). Each sample was examined and photographed with a Zeiss EM 902A (Zeiss, Oberkochen, Germany) operating at 80 kV.

2.4 *Multiphoton Imaging*

A tunable compact mode-locked titanium:sapphire laser (MaiTai, Spectra Physics Inc., Mountain View, California) was expanded by a 1:4 Galilean telescope and coupled to a modified inverted laser scanning microscope (JenLab GmbH, Jena, Germany). The laser has a pulse repetition rate of 80 MHz. The NIR femtosecond laser beam was tightly focused to a diffraction submicron spot. The measurement of the pulse width in the object plane of a 40× oil immersion objective [numerical aperture (NA) 1.3, Zeiss, Jena] was accounted to be 250 fs using an autocorrelator (Autocorrelator Mini, APE Berlin, Germany). Shortly after dissection, ovine pulmonary and aortic heart valve leaflets were investigated at wavelengths of 760 and 840 nm to induce two-photon-excited autofluorescence as well as SHG. The formation of SHG radiation, which occurs at half of the incident laser wavelength, was proved with a filter FB420-10 (Thorlabs Inc., Newton, New Jersey) in front of the detector. A 700-nm short-pass filter (E700SP, Chroma Technology, Brattleboro, Vermont) prevented the scattered laser radiation from reaching the detector. Moreover, this filter blocks UV radiation (transmission range: 390 to 700 nm).

To avoid laser-induced damage, we kept the laser power as low as possible. In fact, when using only the short-pass filter E700SP mean laser powers in the range of 10 to 15 mW were found to be sufficient to obtain high-contrast multiphoton images.

3 Results

3.1 *Histological and Immunohistochemical Staining*

The use of Movat pentachrome stain enabled visualization of the main classes of ECM components in ovine pulmonary and aortic valve leaflet tissues. These include the major proteins collagen and elastin, as well as soluble molecules such as proteoglycans and glycoproteins. A classic formation of the matrix proteins in the four major tissue layers (arterialis, fibrosa, spongiosa, and ventricularis) was clearly visible.[2] Collagen was predominantly detected in the outflow side layers (arterialis-fibrosa), elastin was found at the inflow side (ventricularis) of both valve leaflet tissues. Weigert's resorcin-fuchsin stain enabled additional distinction of the elastic network with single branched fibers (Fig. 1).

Immunohistochemistry visualized no significant differences between the pulmonary and aortic specimens. Collagens I, III, and IV were predominantly detectable at the outflow side layers of both heart valve leaflets. Positive staining for elastin was mainly visible throughout the inflow side layer (Fig. 2).

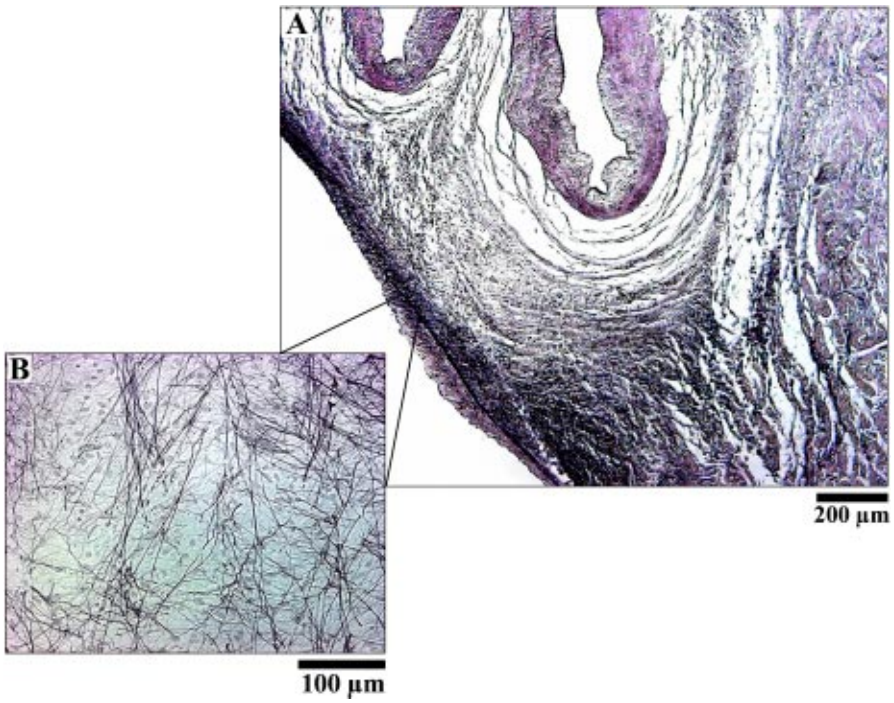

Fig. 1 Bright-field microscopic images of a resorcin-fuchsin-stained (A) longitudinal sectioned and (B) cross sectioned ovine aortic heart valve leaflet to determine the elastic network with single branched fibers (B), predominantly found on the inflow side.

3.2 *Ultrastructural Imaging of Heart Valve Leaflets*

Electron microscopic studies of the outflow side of pulmonary heart valve leaflets showed a collagen-rich matrix, with some single elastin containing structures, very similar to those of the aortic heart valve tissues. The investigation of the inflow side of specimens of both valve tissues revealed an elastic-fiber-rich layer (Fig. 3).

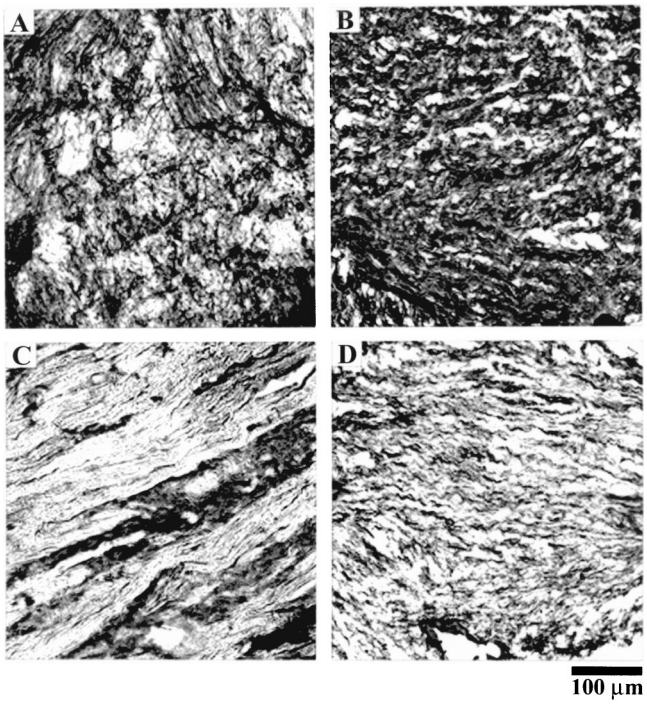

Fig. 2 Immunohistochemical staining of cross-sectioned ovine heart valve tissues. (A) The antielastin antibody reacts with the peripheral zone of mature elastic fibers in the inflow side layer of the valve leaflet. (B) to (D) Inflow side of the leaflet immunostained with antibodies against (B) collagen I, (C) collagen III, and (D) collagen IV.

3.3 *Multiphoton Imaging of Extracellular Matrix Structures*

Fresh, nonsliced and nonstained ovine pulmonary and aortic heart valve leaflet tissues were monitored using the multiphoton system. Matrix components such as collagen and elastin

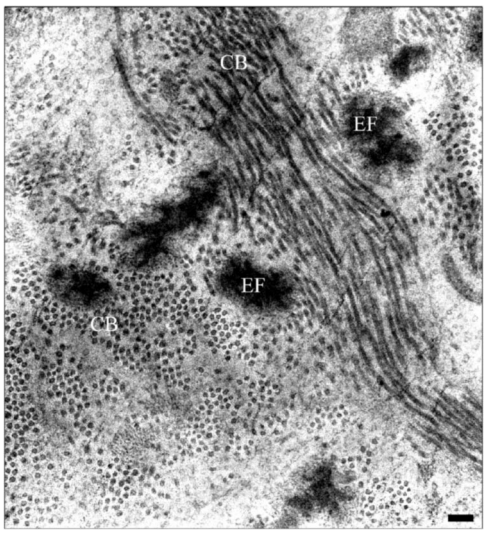

Fig. 3 Electron micrograph of a cross-sectioned ovine aortic heart valve leaflet demonstrating elastic fibers (EF) with close proximity to the surrounding collagen bundles (CB) (scale bar equals 0.33 μm).

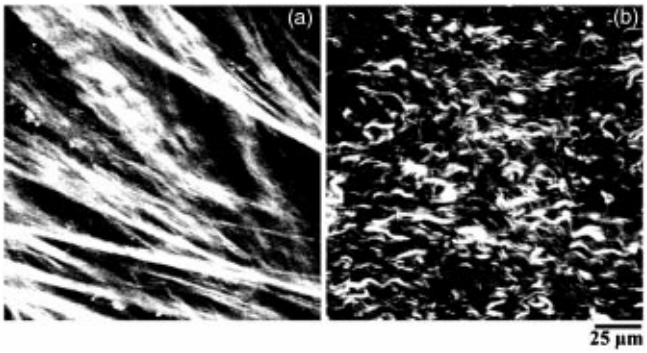

Fig. 4 SHG images of dense bundled collagenous structures on (a) the outflow and wavelike arranged collagen bundles on (b) the inflow side of an ovine pulmonary leaflet, induced with 840-nm NIR femtosecond laser pulses. Images were obtained at tissue depth of 20 μm.

were detected within the working distance of the objective of 120 μm at two different laser excitation wavelengths.

Exposure to laser pulses at 760 nm revealed fibrous structures, most likely autofluorescent elastic fibers. When imaging the same intratissue regions at a higher wavelength at 840 nm, wavelike or dense bundled structures became visible. These structures emitted blue light at 420 nm due to femtosecond-laser-induced SHG processes as proved with the filter FB420-10 [central wavelength (CWL), 420 nm; full width half max (FWHM), 10 nm]. Because it is well known that nonlinear excitation of collagen induces SHG signals, these structures can be attributed to the extracellular matrix protein collagen.

Tissue regions on the outflow side of pulmonary and aortic leaflets showed dense bundled blue-emitting structures, compared to the more wavelike bundled collagenous fibers detected on the elastin-rich inflow side (Fig. 4).

In tissue depths down to 5 to 10 μm, the luminescence was dominated by autofluorescence of elastic fibers (mainly on the leaflet inflow side) and SHG radiation of collagen (on inflow and outflow sides) (Figs. 5 and 6). With increasing tissue depth (>100 μm), the thickness and the frequency of the elastic fibers decreased as well as the amount of collagen.

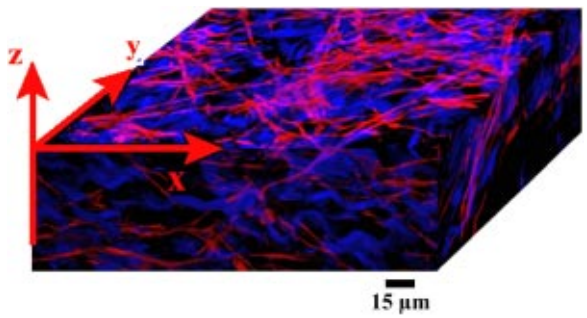

Fig. 5 Three-dimensional reconstruction from a stack of serial autofluorescence/SHG images (z steps=2.5 μm) of an ovine aortic heart valve leaflet, induced with femtosecond laser pulses at 760 nm (red, branched elastic fibers) and 840 nm (blue, wavelike collagenous structures) starting at a tissue depth of 10 μm.

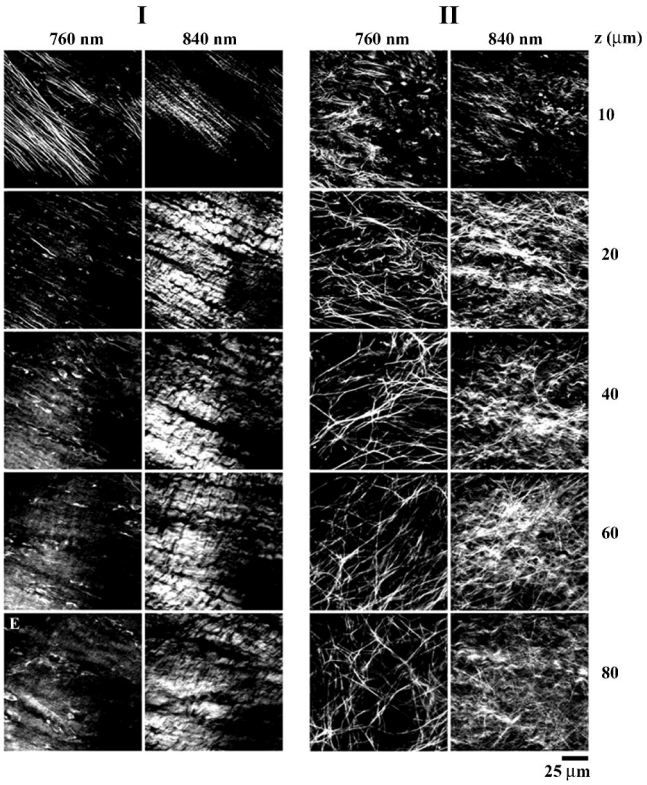

Fig. 6 Two-photon imaging of the (I) outflow and (II) inflow sides of ovine aortic heart valve leaflet tissue, induced with 760- and 840-nm laser pulses. (I) Collagenous bundles are visible at depths of 10 to 80 μm. Just a few elastin-containing structures are shown at depths of 5 to 15 μm. (II) Autofluorescence as well as SHG images demonstrating distinct branched elastic fibers and wavelike collagen bundles throughout the inflow side of the leaflet. The images were chosen out of a stack of representative images.

4 Conclusion

Routine histology, immunohistochemistry, and electron microscopy are traditional and very useful tools in ECM protein identification and general matrix diagnosis, but they are inadequate to predicate the state of the structures within fresh and untreated tissues. In contrast, high-resolution multiphoton imaging with NIR ultrashort laser pulses is an attractive method to study the development and morphology of intact intratissue ECM structures.[18–20] For this purpose, NIR femtosecond multiphoton laser imaging was used in this paper to visualize the fibrous ECM components such as elastin and collagen within intact ovine aortic and pulmonary heart valve leaflets without any fixation, embedding, or staining procedures. Optical sectioning enabled the complete 3-D reconstruction of the leaflet architecture in its normal hydrated state.

To distinguish between the different ECM structures, two laser excitation wavelengths of 760 and 840 nm were employed. Using a wavelength of 840 nm two-photon-excited autofluorescence was induced. Furthermore, a strong signal was obtained by the nonlinear process called SHG, which implicated the collagenous origin of the visualized structures. Therefore, it is possible to image collagen-containing matrix elements almost selectively by 840 nm. In contrast, when using a wavelength of 760 nm, autofluorescent structures were observable dedicated to both ECM proteins—elastin and

collagen[21]—whereas the autofluorescence signal from collagen was conspicuously weaker than the signal received from elastin. However, using the short-pass filter E700SP, which has no transmittance in the UV, only autofluorescence signals and no SHG were detected. As shown in the previous literature, these autofluorescent fibrous structures can be attributed to elastin.[22]

The resulting high-resolution 3-D autofluorescence and SHG images showed that a distinct system of elastic fibers was mainly found at the inflow side of the leaflets, whereas dense bundled collagenous structures were predominantly detectable at the outflow side of the ovine aortic and pulmonary heart valve leaflets. These findings were verified by comparable results achieved by the use of routine bright-field light microscopy and electron microscopy.

NIR multiphoton laser imaging provide attractive advantages over conventional fluorescence techniques and can be employed as a novel noncontact optical tool for 3-D-resolved ECM component imaging and tissue state diagnosis. In the near future, NIR microscopy has the potential to become one of the favored methods in biotechnology, biomedical science, and tissue engineering as a fast procedure for the investigation of ECM fiber quality as well as to diagnose a variety of diseases including ECM-related dermatological disorders or affected cardiovascular structures.

The promising results of this basic study on vital nonstained heart valve tissues are the premise for ongoing investigations of diseased heart valves and tissue engineered cardiovascular constructs.

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