Nonlinear optical imaging of extracellular matrix proteins

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Abstract

Over the last 2 decades, nonlinear imaging methods such as multiharmonic imaging microscopy (MHIM) have become powerful approaches for the label-free visualization of biological structures. Multiharmonic signals are generated when an intense electromagnetic field
propagates through a sample that either has a specific molecular orientation or exhibits certain physical properties. It can provide complementary morphological information when integrated with other nonlinear optical imaging techniques such as two-photon excitation (TPE). Here, we present the necessary methodology to implement an integrated approach for multiharmonic and TPE imaging of the mouse aorta using a commercial two-photon microscope. This approach illustrates how to differentiate the microstructure of the mouse aorta that are due to collagen fibrils and elastic laminae under 820 and 1230nm excitation. Our method also demonstrates how to perform multiharmonic generation by reflectance of the forwardly propagating emission signal. The ability to visualize biological samples without additional genetically targeted or chemical stains makes MHIM well suited for studying the morphology of the mouse aorta and has the potential to be applied to other collagen and elastin-rich tissues.

1 INTRODUCTION

The utilization of multiharmonic imaging microscopy (MHIM) to visualize biological structures has become more prevalent over the past 2 decades. This approach, based on a nonlinear optical (NLO) effect known as multiharmonic generation (MHG) has been established as a viable microscope imaging contrast mechanism for the visualization of in situ cellular and tissue function without the need for chemical or biochemical labels (Campagnola & Loew, 2003; Freund, Deutsch, & Sprecher, 1986; Tai, Kung, Yu, Huang, & Chan, 2007; Weigelin, Bakker, & Friedl, 2016). Owing to the necessity of using lower energy infrared wavelengths for harmonic generation, the process facilitates both optical sectioning and deeper penetration into turbid milieu. This provides inherent three-dimensional (3D) structural information in both in vitro and ex vivo sample types (Bayan, Levitt, Miller, Kaplan, & Georgakoudi, 2009; Osman et al., 2013; Rehberg, Krombach, Pohl, & Dietzel, 2011). Typically, second-harmonic generation (SHG) requires a material to have a specific molecular orientation in order for the incident light to be frequency doubled. However, some biological materials can be highly polarizable, and assemble into fairly ordered, large noncentrosymmetric structures. Biological materials such as collagen (Freund et al., 1986; Friedl, Wolf, von Andrian, & Harms, 2007) and microtubules (Kasischke & Hyman, 2003) can produce SHG signals. Furthermore, the presence of water–lipid or water–protein interfaces (Chen & Sun, 2009; Jay et al., 2015; Tai et al., 2007) can elicit third-harmonic generation (THG), or frequency-tripled signals. Because these processes are nonlinear in nature and do not involve molecular excitation (as in the case of fluorescence microscopy) the effects of phototoxicity and photobleaching are not a concern (Brakenhoff, Squier, Wilson, & Müller, 1998; Débarre, Olivier, Supatto, & Beaurepaire, 2014). With these obvious advantages, MHIM microscopy has been widely employed in studies of the extracellular matrix (Weigelin, Bakker, & Friedl, 2012; Wu, Hsieh, Tsai, & Liu, 2015), inorganic structures (Chu et al., 2005; Gannaway & Sheppard, 1978; Hellwarth & Christensen, 1974; Marangoni, Lobino, & Ramponi, 2006), and cellular
membranes/organelles in both fixed and living specimens (Chen & Sun, 2009; Rehberg et al., 2011; Weigelin et al., 2012).

SHG, also known as frequency doubling, is a NLO effect that occurs when an intense electromagnetic field passes through a material with a noncentrosymmetric molecular structure (Campagnola & Loew, 2003; Campagnola, Wei, Lewis, & Loew, 1999). Second-harmonic light emerging from a SHG material is exactly half the wavelength (i.e., frequency doubled) of the light entering the material. Two incident photons are combined into one photon (second harmonic) with a higher energy, which propagates in the same direction as the incident photons. Typically, an inorganic crystal such as lithium niobate (LiNbO₃) potassium titanyl phosphate (KTP = KTiOPO₄) or lithium triborate (LBO = LiB₃O₅) are used to produce visible light from near-infrared lasers or UV light from visible lasers in the optics industry. Early research by Hellwarth and Christensen (1974) and Gannaway and Sheppard (1978) suggests that the observation of SHG induced by biological specimens under microscope illumination is theoretically possible, assuming illumination with a pulsed, high peak-power laser source. However, second-harmonic imaging was not attempted on a biological sample until the mid-80s when the dipolar formation of collagen fibers in the rat-tail tendon was elucidated (Freund et al., 1986). This study demonstrated the potential of second-harmonic imaging for the visualization of biological ultrastructure based on the molecular organization of noncentrosymmetric macromolecules such as collagen. Types I and II collagen, which form aligned fibers, are primarily found in skin, tendon, vascular ligature, bone, and cartilage (Chen, Nadiarynk, Plotnikov, & Campagnola, 2012). Being able to image fibrillar collagen without the addition of an exogenous fluorescent label broadly expands the application of this technique. In addition, the other protein-based macromolecules such as the acto–myosin complex and tubulin are also SHG active (Campagnola et al., 2002; Mohler, Millard, & Campagnola, 2003). Owing to the number of SHG-active macromolecules, second-harmonic imaging has allowed the extraction of a substantial amount of structural information on cancerous cells and general tissue organization which has solidified its impact in biomedical research (Jay et al., 2015; Pfeffer, Olsen, Ganikhanov, & Légaré, 2011; Wu et al., 2015).

THG known as frequency tripling also results from the summation of photons induced by specific physical properties of the sample (Brakenhoff et al., 1998; Weigelin et al., 2016). THG triples the energy and generates a single photon with a wavelength one-third of the incident photons. Unlike SHG though, THG occurs mainly at interfaces between materials of differing excitability, such as the refraction index mismatch between water–lipid or water–protein interfaces, or resonant enhancement by chromatic materials such as hemoglobin. Since the presence of a non-centrosymmetric structure is not required for THG, its applications are more wide ranging than SHG. These have included studying collagen and elastic fibers (Rehberg et al., 2011), calcified teeth (Chen, Hsu, & Sun, 2008), cellular and nuclear membranes (Aptel et al., 2010; Chen, Wu, & Sun, 2009), adipocytes (Tsai et al., 2013), and neurites (Farrar, Wise, Fetcho, & Schaffer, 2011; Lim et al., 2014). In addition, this lower specificity makes manipulation of the THG signal easier for
visualizing separate structures. For example, altering the axial position of the focal volume allows for the illumination of different cellular components in Drosophila embryos thus facilitating discrimination of the THG signal produced by external membrane vs internal organelles (Aptel et al., 2010; Mahou et al., 2011). Another example is changing the angle between the sample interface and the incident laser can facilitate detection of different orientations of fibrillar collagen (Cheng & Xie, 2002; Weigelin et al., 2016). All these features contribute to the increased versatility THG microscopy enjoys as opposed to its SHG relative.

A common setup for a MHIM system can be routinely achieved by proper modification of a commercial laser-scanning NLO microscope equipped with a titanium sapphire mode-locked laser as the illumination source. Acquisition of the SHG signal (with a frame rate and pixel density similar to that of a laser-scanning confocal microscope) can be routinely achieved by the selection and installation of an appropriate dichroic mirror and band-pass filter combination in front of the photodetector. However, for THG, both the illumination objective lens and detection light path need to be optimized for the highest sensitivity. The availability of high-quantum efficiency gallium arsenide phosphide (GaAsP) photomultipliers drastically improves signal-to-noise ratios and thus lowers the barrier for THG imaging on biological specimens. Given its increased popularity, integration of nonlinear SHG and THG imaging with two-photon excited fluorescence (TPEF) has become a common practice that offers complementary information. For example, in myofibers, SHG signals mainly result from myosin that is localized in the -A-band of the sarcomere, while THG signals result from the actin-rich I-bands that reside between sarcomeres (Rehberg et al., 2011; Rehberg, Krombach, Pohl, & Dietzel, 2010; Weigelin et al., 2012). Conversely, 3D SHG/THG imaging of a collagen fiber network, data illustrate complementary patterns between the SHG and the THG signals that result due to the orientation of the fibrillar collagen (Weigelin et al., 2016).

In this chapter, we will focus on describing the theoretical principles of MHIM, the practical implementation of the approach to image cross-sections of mouse aorta along with the necessary steps for suitable sample preparation. One of the first imaging paradigms that paired SHG with two-photon excitation (TPE) was that of Zoumi, Lu, Kassab, and Tromberg (2004). They made use of an excitation wavelength of 800nm and successfully separated SHG and TPEF signals from coronary artery in ranges of 390–410 and 500–540nm. This approach allowed the capture of the morphology of collagen fibrils in the outer layer (adventitia) by SHG and elastin fibrils in the middle layer (medial) by TPEF. This approach was later modified for imaging arterial microstructures under 860nm excitation (Boulesteix et al., 2006). We describe a further modification of the earlier approach by demonstrating how to induce and capture the augmented third-harmonic signal by reflectance of the forwardly propagating THG signal by a dielectric mirror. Such a methodology has been used previously to enhance MHIM signals as it serves to improve the signal-to-noise ratio on the backward scattered detector (Rehberg et al., 2010). We have utilized this
paradigm for evaluating the microstructure of the mouse aorta. Since the polymeric materials collagen and elastin are two well-characterized components present in blood vessels that aid in maintaining healthy function, MHIM paired with TPEF imaging serves as a powerful, label-free microscopy method for the study of the morphology and pathology of the vascular system.

2 METHODS

2.1 THEORETICAL PRINCIPLES OF MHG

The nonlinear polarization of a given material can be expressed by a Taylor series expansion of the dielectric polarization density (dipole moment per unit volume) $P(t)$ at time $t$ in terms of electrical field $E(t)$:

$$P(t) = \varepsilon_0 \left( \chi^{(1)} E(t) + \chi^{(2)} E^2(t) + \chi^{(3)} E^3(t) + \cdots \right)$$

where the coefficients $\chi^{(n)}$ are the $n$th order nonlinear susceptibilities of the material, $\chi^{(1)}$ describes the normal absorption and reflection processes; $\chi^{(2)}$ describes SHG, sum, and difference frequency generation; and $\chi^{(3)}$ describes THG, multiphoton absorption, and light scattering (Barad, Eisenberg, & Horowitz, 1997; Campagnola et al., 1999).

SHG, which dominates the second-order term, is highly restricted to materials with noncentrosymmetric molecular organization. The susceptibility tensor of second-order $\chi^{(2)}$ is a bulk property that is related to molecular hyperpolarizability by:

$$\chi^{(2)} = N_s \langle \beta \rangle$$

where the $N_s$ is the molecular density of the material and $\langle \beta \rangle$ is expectation value of the molecular orientations in the material, i.e., the orientational average. The requirement for noncentrosymmetric symmetry is necessary to prevent $\langle \beta \rangle$ from vanishing for an isotropic distribution of dipole moments, i.e., to keep $\chi^{(2)}$ nonzero. Type-I/II collagen and acto–myosin complexes are the two main macromolecular assemblies that satisfy this requirement. In contrast, nonfibrillar collagen, such as type-IV, fails to produce any appreciable SHG signal due to the lack of a noncentrosymmetric structure. In the case of THG, however, the signal is generated from the third-order susceptibility factor ($\chi^{(3)}$). Thus in tissues, THG occurs predominantly at water–lipid or water–protein interfaces, or within media that exhibits significant optical heterogeneity, such as lipid-containing organelles and cells, the myelin sheath surrounding nerve fibers as well as cellular membranes and intracellular vesicles. Unlike SHG, which is highly specific to macromolecular structures that are noncentrosymmetric, the intensity of the generated THG signal is highly dependent on the interaction between the focal volume and interface of changing refractive index or $\chi^{(3)}$. 
The energy-level schema of MHG and TPE are depicted in the Jablonsky diagram shown in Fig. 1A. In the process of TPE, two lower energy photons (typically in the infrared region of the electromagnetic spectrum) near-simultaneously excite a fluorophore from its ground electronic state \( S_0 \) to its first- and second-excited electronic states.
states ($S_1$ and $S_2$). This fluorophore, by virtue of intramolecular vibrational redistribution dissipates some of this excited state energy as heat but releases the majority of it by emission of a photon of light (typically in the visible region of the electromagnetic spectrum) as it relaxes back to its ground electronic state. Instead of the characteristic absorption and emission processes that are seen in TPE, SHG, and THG are induced by the presence of a very intense field that results in the production of a nonlinear polarization inside the focal volume. Because the SHG and THG signals are coherent waves at double or triple the incident frequency, molecules that contribute to either the SHG signal or THG signal are no longer considered to be point emission sources as in the case of a fluorescing object. As such, the direction of the nonlinear harmonics critically depends on the spatial distribution of the molecules as well as the dipole moment of the driving field. Thus the generated harmonics propagate in a direction that is parallel to the incident beam.

In order to settle on an incident wavelength for MHIM, one must find the balance between light scattering and absorption, especially in the case of THG imaging. From a theoretical perspective, longer wavelengths (>1350nm) serve to reduce light scattering, but they suffer from a reduced tissue penetration due to the absorption effects of water. Conversely, shorter wavelengths (<1100nm) would induce THG in the ultraviolet (UV) range, which is highly scattered by tissue and has low transmission through optical glass components. Hence, the ideal wavelength to induce THG would be by using infrared excitation (1200–1300nm) thus generating THG signal in the blue region of the electromagnetic spectrum (400–450nm). With this particular wavelength, one can also induce SHG simultaneously in the red region of the electromagnetic spectrum (600–650nm) (Jay et al., 2015, 2008; Rehberg et al., 2011; Wu et al., 2015). In our method described below, we have chosen a wavelength of 820nm for SHG induction and 1230nm for THG induction (Fig. 1B). This enables the simultaneous acquisition of the MHG/TPE signals on a pair of nondescanned detectors (NDDs), which are equipped with a dichroic mirror and two band-pass filters, which serve to separate and capture the spectral ranges of 380–430 and 580–670nm, respectively.

2.2 IMPLEMENTING MHIM

2.2.1 Instrumentation

We implemented this methodology on a commercial Zeiss LSM 880 NLO two-photon microscope system installed on an Axio Observer Z.1 inverted microscope frame (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a pulsed, tunable infrared laser source (Chameleon Discovery, Coherent Inc., CA, USA) (Fig. 2A). The Chameleon Discovery laser generates emission in the range 680–1300nm with a pulse duration of ~100 fs at a repetition rate of 80 ± 0.5 MHz. The output at 1230nm was ~1100 mW. The average intensity at the sample plane was 8.96 ± 0.09 mW at 820nm and 35.8 ± 0.07 mW at 1230nm. A Plan-Apochromat 63 × 1.4NA oil immersive objective lens with a working distance of 0.19 mm was used for imaging (Carl Zeiss Microscopy GmbH, Jena, Germany). During MHIM, both SHG and THG signals were detected by two nondescanned gallium arsenide phosphide photomultiplier tubes (GaAsP PMTs) (Zeiss BiG-2, Carl Zeiss Microscopy GmbH, Jena,
FIG. 2
Multiharmonic imaging microscopy. (A) Schematic overview of Zeiss LSM 880 NLO imaging system. The tunable femtosecond laser is equipped for NLO imaging on a laser scanning system, controlled by the galvano mirror. Emission fluorescent or harmonic signals propagated backward to the high-sensitivity PMTs in the nondescanned detector (NDD) or the spectral PMT in the descanned detector. Enhancement by the dielectric mirror reflection can be optional. (B) The reflection of SHG and THG signals when mirror is presence. This setup was adapted from a method described by Rehberg, M., Krombach, F., Pohl, U., & Dietzel, S., 2010. Signal improvement in multiphoton microscopy by reflection with simple mirrors near the sample. Journal of Biomedical Optics, 15(2), 026017. https://doi.org/10.1117/1.3374337.
Germany). The incoming light to the BiG-2 detector assembly was split by a 468 nm short pass dichroic mirror and filtered by two band-pass filters, one in the blue (380–430 nm) and one in the far-red (580–670 nm) (Semrock, NY, USA) channels. To confirm the spatial pattern of autofluorescence elicited by TPE, we applied 820 nm laser excitation and detected the fluorescence emission in multiple ranges using a descanned GaAsP spectral detector (QUASAR array) equipped within the Zeiss LSM 880 NLO scan head. To enhance the MHG signals, a broadband dielectric mirror (BB2-E02, 400–750 nm, Thorlabs Inc., NJ, USA) was placed on the back of sample slide to reflect the forward scattered signal to the backward facing detectors (Fig. 2B).

2.2.2 Image acquisition

Single image planes and z-stack image data were acquired using the ZEN software (ZEN 2.3 SP1 Black, Carl Zeiss Microscopy, Jena, Germany). To enhance the signal-to-noise ratio, the scan speed was set to 1, which corresponds to a pixel dwell time to 65.94 μs/pixel, and the frame size was set to either 512 × 512 with an optical zoom factor of 1.5 × (0.18 μm/pixel) or 1024 × 1024 without no optical zoom (0.13 μm/pixel). For multiharmonic imaging using the NDD, increasing the digital gain to 8.0 significantly improved the quality of the acquired THG image. However, care must be taken when using digital gain as it is simply a multiplication factor. While the signal intensity will be enhanced, so will any noise present in the image. In contrast, the SHG and the TPE signals were much stronger than the THG signal, so the use of digital gain was not required. Also, due to the low signal obtained from THG, the higher dynamic range provided by a 16-bit depth acquisition resulted in more robust data for postprocessing and analysis. The voltage of GaAsP PMTs was set to 900 and 600 V for the acquisition of THG and SHG/TPE signals, respectively. Owing to the high sensitivity of the GaAsP PMTs, it is especially important to maintain a dark imaging environment with proper blackout material. Furthermore, the slides were secured onto the slide holders by an orthodontic wax to prevent any lateral drift caused by the weight of the dielectric mirror. For TPE spectral imaging using the descanned QUASAR spectral detector, the voltage was set between 700 and 900 V with digital gain set to 1. For all imaging, no digital offset was applied.

The theoretical resolution of an optical system can be estimated by the Rayleigh criterion:

\[
    d_{\text{min}} = 0.61 \times \frac{\lambda}{\text{NA}_{\text{objective}}}
\]

where \(\lambda\) is the wavelength, \(\text{NA}_{\text{objective}}\) is the numerical aperture of the objective lens, and \(d_{\text{min}}\) is the smallest spatial distance that can be resolved. With a Plan-Apochromat 63 × 1.4NA oil immersion objective lens (Carl Zeiss Microscopy, Jena, Germany) and the known wavelength from MHG, \(d_{\text{min}}\) is 174 nm in the range of SHG and THG induced at ~800 and ~1200 nm, respectively, and 261 nm for the TPE autofluorescence emission at 600 nm. No oversampling was considered as the image pixel sizes were greater than a factor of 1/3 to 1/2 of \(d_{\text{min}}\).
2.2.3 Image and data processing
All image data were saved as both proprietary Zeiss CZI as well as exported to TIFF format for subsequent processing in ImageJ (Schindelin, Rueden, Hiner, & Eliceiri, 2015) and/or Fiji (Schindelin et al., 2012). The plugins necessary for visualization purposes include Bio-Formats (Linkert et al., 2010) and ClearVolume (Royer et al., 2015). All figures included in this chapter were created using the Adobe Creative Cloud Illustrator App (Adobe, CA, USA).

2.3 SAMPLE PREPARATION

2.3.1 Materials
1. Coverslip—Microscope cover glasses, size: 22 × 50mm, thickness: #1.5 (160–190μm), Cat. no. 12-544-D (Thermo Fisher Scientific, MA, USA).
2. Slide—Microscope slides, precleaned, Cat. no. 12-550-003 (Thermo Fisher Scientific, MA, USA).
3. Fixative—4% Paraformaldehyde (EM grade) in phosphate-buffered saline (PBS).

2.3.2 Sample collection and fixation
Mice at 2 months of age (maintained on a 12-h/12-h light/dark cycle with food and water ad libitum) were used in preparing the aorta sections. Animals were first anesthetized with Avertin (Sigma-Aldrich Co., MO, USA), and then the heart and lungs were then exposed by opening the chest cavity. The ascending aorta or carotid artery was removed after several washes in PBS. Once dissected, an identified target region in the tissue was first washed in the PBS to remove excess blood prior to fixation in 4% paraformaldehyde inside of microcentrifuge tubes stored at 4°C for 20h, followed by two washes in PBS for 15 min each. Samples were then dehydrated sequentially in a graded ethanol series (30%, 50%, and 70%) for 30 min each, and embedded in paraffin with a Leica HistoCore Arcadia H (Leica Biosystems Inc., IL, USA). Tissue sectioning was performed using a manual rotary microtome (RM2235, Leica Biosystems Inc., IL, USA).

3 IMAGING THE MICROSTRUCTURE OF MICE AORTA USING MHIM
The aorta is the main artery that is responsible for the systemic circulation of oxygenated blood from the heart to the circulatory system. In order to sustain the large volume of blood that flows through it, the aorta consists of sheets (lamellae) of elastin and bundles of fibrous collagen, which serve to provide and maintain the elasticity of the arterial wall. Any change in the quantity or quality of the matrix components will result in both a mechanical and functional alteration, which potentially develops into disease pathology. To date, the architecture of the aorta has been most commonly...
studied using pigment-based stains such as Sirius Red and Verhoeff’s solution (Boulesteix et al., 2006; Jiao et al., 2017) which target elastin and collagen. Such staining approaches are easy to perform but yield a product that can only be visualized using brightfield optical microscopy, which lacks the ability to optically section. Previous work have shown that by utilizing a transgenic animal expressing fluorescent reporters or by combining SHG with TPE microscopy under a singular excitation wavelength (in the range 800–860nm) it is possible to acquire the 3D spatial organization of both collagen and elastin (Boulesteix et al., 2006; Zoumi et al., 2004). The strong autofluorescence signal from elastin and SHG signal that is induced by fibrillar collagen can be captured simultaneously and used to elucidate the microstructure of the aorta. We have recapitulated this combined SHG/TPE paradigm in our commercial NLO imaging system. We also demonstrate the possibility for a combined SHG/THG/TPE imaging approach using a mirror-based augmentation methodology.

3.1 DETECTING MIRROR-AUGMENTED MHG SIGNALS FROM THE MOUSE AORTA

As described above, MHG signals typically propagate in the direction of the incident beam, thus forward-scattered SHG and THG photons are substantially more prevalent than backward-scattered photons. To successfully detect MHG signals on a detector in the backward-scattered direction, a straightforward method is to enhance the signal by reflecting forwardly propagating signal back to the backward-scattered detector with a simple mirror. Such a methodology was first described in the imaging of mouse cremaster muscle by Rehberg et al. In their experiment, SHG and THG signals were increased by factors of 9.6 and 7.6 times, respectively, by the presence of an aluminum-coated coverslip located behind the sample plane (Rehberg et al., 2010). To reproduce this method on our system, we placed a broadband dielectric mirror on top of the sample, which generated a significant enhancement of the MHG signal. Using 820nm light, SHG signal induced by fibrillar collagen in the adventitia of a mouse aorta was easily detected in the spectral range 380–430nm (Fig. 3A). Despite this signal already being sufficient for detection, its intensity increased approximately twofold to threefold in the center of the collagen bundles (Fig. 3B). In contrast, the induced THG signal was so low without the presence of the reflecting mirror (Fig. 3C), that it increased up to sevenfold when the forward-scattered signal was reflected (Fig. 3D). The TPE autofluorescence that was detected by in the spectral range 600–750nm did not exhibit significant enhancement upon mirror augmentation irrespective of 820nm or 1230nm excitation. The only increase in autofluorescent signal originated from elastic laminae under 820nm excitation, which was clearly separated from other autofluorescent sources such as smooth muscle cells containing NAD(P)H and flavin molecules. Interestingly, the same enhancement was not observed under 1230nm excitation.

Note to reader: The broadband dielectric mirror used for reflection of the forward-scattered MHG signal was placed directly on top of the sample slide.
FIG. 3
The mirror-augmented MHG signals from mice aorta. (A) Representative cross-sectional images of the SHG/TPE signals at 820 nm from mice aorta, with and without the reflection of forward light propagation and (B) the intensity profiles inside the adventitia or across the wall (white dash line). The intensity of SHG was increased twofold to threefold in the center of collagen bundle with the dielectric mirror (arrows). However, the augmentation was not homogenous and presented lower degree of enhancement on location where the SHG was already strong without mirror. In contrast, the autofluorescence signals show nearly no difference with and without mirror except the elastin lamina (arrows). (C) Representative cross-sectional images of the THG/TPE signals at 1230 nm from mice aorta and (D) the intensity profiles across the wall (white dash line). The enhancement of THG was from twofold to sevenfold (arrows), but no change of TPE signals was shown. A: adventitia (outer layer), M: media (middle layer), and L: lumen. Scale bars: 20 μm.
As such, all visible light between 400 and 750nm was reflected. Since detection of MHG signals requires high sensitivity, it is essential that ambient illumination in the imaging area is kept to an absolute minimum.

### 3.2 SPECTRAL COMPARISON OF MHG AND TWO-PHOTON EXCITED AUTOFLUORESCENCE HIGHLIGHTS DIFFERENCES IN TISSUE MICROSTRUCTURE

Both the mirror-augmented MHG and TPE signals were compared using the NDD GaAsP PMTs and the GaAsP QUASAR spectral detector in the same spectral bands. First, the SHG signal was captured successfully by both the external NDD and the internal descanned spectral detector and showed, as expected, a highly correlated pattern. However, the optical loss that the emission suffered on the descanned light path to the spectral detector was sufficient to preclude detection of the expected, albeit much weaker THG signal at 1230nm excitation (Fig. 4A and B). The images captured at 820nm excitation using an NDD coupled with a red band-pass filter (580–670nm) and those captured by the descanned spectral detector are quite similar. The spatial patterns of lamellar structures are consistent with those previously reported (Boulesteix et al., 2006; Zoumi et al., 2004). The observed autofluorescence potentially being contributed by desmosine and isodesmosine crosslinks between polypeptide chains, which can be visualized at ease with an excitation wavelength in the range 800–860nm. However, the autofluorescent signature was not limited to lamellar structures, in fact 820nm excitation also elicited autofluorescence from the interlaminar spaces as well as the adventitia, which are highlighted in the green and red regions of the spectra using the spectral detector. However, using 1230nm excitation showed a stark difference in the autofluorescence spectrum with the majority of the previously observed green signal under 820nm being completely absent. This phenomenon served to significantly increase the contrast of the elastic laminae.

Two-photon excited autofluorescence spectra from three different aortic microstructures, the adventitia, the elastic laminae, and the interlaminar space were analyzed (Fig. 4C). Regions of interest defined in the spectral data stacks obtained at both 820 and 1230nm excitation, revealed that the elastic laminae exhibit a peak in their autofluorescent spectra in the range 660–670nm that was absent from regions defined in both the adventitia and the interlaminar spaces. This supports the hypothesis that lamellar structures preferentially contribute to the autofluorescence signal in the far-red regions of the electromagnetic spectrum. At 820nm excitation, strong autofluorescence signal was observed in the spectral range 530–580nm in the regions of interest highlighting the adventitia. This signal most likely is contributed from either smooth muscle cells containing NAD(P)H and/or flavin compounds. These species may also contribute the moderate autofluorescence present in the elastic laminae and the interlaminar spaces. These signals, however, were exhibited at 1230nm excitation, which served to significantly increase the relative contrast of lamellar structures.
FIG. 4
See figure legend on next page.
3.3 VISUALIZING THE MICROSTRUCTURES OF MOUSE AORTA USING NONLINEAR OPTICAL MICROSCOPY

Because both MHG and TPE happen inside the same spatial excitation volume, each technique is inherently able to optically section to generate 3D data. By acquiring the MHG/TPE signals under 820 nm excitation in 3D, it is possible to visualize both the ribbon-like collagen bands and lamellar elastin structures, thus providing detailed information on the microstructure of the aortic wall (Fig. 5A, top). Images acquired at different axial depths within the sample allow a 3D reconstruction to be built (Fig. 5B, left), which highlights the distinct structures of elastin and collagen present in the aortic wall. The same principle can be applied to the THG/TPE signals under 1230 nm excitation (Fig. 5A, bottom, and Fig. 5B, right). In contrast to the specificity of SHG signals that arise from fibrillar collagen, the source of THG in the mice aorta is not well known. Potential sources are smooth muscle, endothelial cells, fibroblast-like cells, and extracellular matrix components. Because of the inherent complexity of the microstructure present, heterogeneous THG signals were shown in both adventitia and media, and especially in the interlamellar spaces (Fig. 6A). In the lumen, the resonant enhancement of the THG signal due to the presence of hemoglobin was observed and allowed the observation of the morphological shape of blood cells, consistent with previous reports (Chang, Yu, & Sun, 2010; Rehberg et al., 2011). It is clear though that further investigation will be required in order to be able to identify the nonspecific sources of THG signals. In contrast, the TPE signal under 1230 nm excitation shows both high contrast and high specificity which aids in subsequent reconstruction and segmentation. This added benefit could have potential benefits for investigating the microstructure of elastic laminae. For example, the surface of elastic laminae contains fenestrae, which are small holes on the order 1–10 μm in diameter, which also vary between different age and species. Their exact purpose and physiological function...
is not well known, but has been linked in facilitating nutrient transport between cells in the interlaminar spaces (Wong & Langille, 1996), and could potentially be aberrant in diseases like Marfan syndrome (López-Guimet, Andilla, Lozad-Alvarez, & Egea, 2017). In our experimental system, we successfully observed
fenestrae with high contrast using the autofluorescence of elastin under 1230nm excitation (Fig. 6B). Taking into consideration the additional penetration of 1230nm vs 820nm light into scattering milieu, this could be useful for studying microstructural changes that arise when the elastic laminae are disrupted.

**FIG. 6**
Structural details delineated by MHIM and TPE. (A) Representative THG/TPE images from adventitia, media, and lumen. Scale bars: 10μm. (B) Fenestrae (arrows) were identified under the 3D MHIM in the stacks of four images acquired every 1.25μm. Scale bars: 10μm.
4 CONCLUSIONS

In this method, we have presented an integrated approach to allow investigators to differentiate the structural components of mouse aortic wall by combining two NLO techniques: MHIM and TPE microscopy. Under 820 nm excitation, SHG and TPE signals that primarily correspond to collagen and elastin can be spectrally separated and exhibit identical patterns in the adventitia, the elastic laminae, and the interlaminar spaces. These results are consistent with previously published work that suggest that the SHG/TPE imaging can be used as a replacement for conventional histological methods for identifying microstructures in the mouse aorta (Boulesteix et al., 2006; Zoumi et al., 2004). The tunable pulsed femtosecond laser is a powerful tool for us to examine the THG/TPE imaging paradigm on the same samples. We have demonstrated that under 1230 nm excitation, the elastic laminae exhibit higher contrast in TPE imaging. This could have an added benefit as images with enhanced signal-to-noise ratios make the postprocessing and analysis of fine structures such as fenestrae more feasible. In addition, our approach is not limited to blood vessels, rather it may facilitate the study of other collagen- or elastin-rich tissues such as elastic cartilage, skin, or the bladder.

Furthermore, we achieved THG imaging with the same infrared laser wavelength (i.e., 1230 nm) on cross-sections of mouse aorta by reflecting the forwardly propagating THG signal to the backward detector. To the best of our knowledge, this is the first report showing the acquisition of THG signals obtained from mouse aorta tissue. However, further investigation is required to successfully identify the source of THG signals since refractive index mismatching can significantly occur inside the microenvironment of blood vessels. THG signals most likely originate from a combination of smooth muscle and/or additional extracellular matrix components that may alter under a specific pharmacological treatment or genetic modification. Therefore, computational approaches may be necessary to evaluate the spatial pattern of the THG signals, which have been used previously to characterize the microarchitecture of collagen fibrils (Bayan et al., 2009; Boudaoud et al., 2014; Osman et al., 2013). Finally, by integration of SHG/TPE and THG/TPE imaging data with other labeling techniques, such as immunofluorescence staining or fluorescent fusion proteins, it will be possible to provide a more complete morphological picture and help in understanding more about the physiological functions and pathological progress of disease in the mouse aorta.

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REFERENCES


CHAPTER 3 Nonlinear optical imaging of extracellular matrix proteins


