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Multimodal nonlinear optical imaging of collagen arrays

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Abstract

We report multimodal nonlinear optical imaging of fascia, a rich collagen type-I sheath around internal organs and muscle. We show that second harmonic generation (SHG), third harmonic generation (THG) and Coherent anti-Stokes Raman scattering (CARS) microscopy techniques provide complementary information about the sub-micron architecture of collagen arrays. Forward direction SHG microscopy reveals the fibrillar arrangement of collagen Type I structures as the main matrix component of fascia. SHG images detected in the backward direction as well as images of forward direction CARS microscopy show that the longitudinal collagen fiber bundles are further arranged in sheet like bands. Forward THG microscopy reveals the optically homogeneous content of the collagen sheet on a spatial scale of the optical wavelength. This is supported by the fact that the third harmonic signal is observed only at the boundaries between the sheets as well as by the CARS data obtained in both directions. The observations made with THG and CARS microscopy are explained using Atomic Force Microscopy images.

Keywords

Collagen; Nonlinear Optical Microscopy; Atomic Force Microscopy

1. Introduction

Multiphoton laser scanning microscopy is a powerful technique for high spatial resolution ($\sim 1\mu\text{m}$) and deep tissue imaging ($\sim 1\text{mm}$) ([Denk et al., 1990], [Helmchen and Denk, 2005], [Svoboda and Yasuda, 2006] and [Dunn and Young, 2006]). Using femtosecond laser sources that induce two-photon absorption followed by fluorescence emission, multiphoton fluorescence microscopy is now well adapted and a key technique for imaging cellular components as well as extracellular matrices of tissues within thick sections ([Centonze and White, 1998], [Zoumi et al., 2002] and [Zipfel et al., 2003]) or living animals ([Baksai et al., 2003], [Stosiek et al., 2003] and [Spires et al., 2005]).

The application of two-photon induced fluorescence is limited by phototoxicity and photobleaching due to electronic excitation. During the last few years, it has been demonstrated that SHG microscopy can be combined easily with multiphoton fluorescence technique. SHG microscopy is highly sensitive to the presence of biological structures with

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noncentrosymmetric molecular organization ([Roth and Freund, 1979] and [Campagnola and Loew, 2003]). SHG microscopy has been successfully used to image structural protein arrays, such as collagen Type I and –III ([Zoumi et al., 2002], [Brown et al., 2003], [Williams et al., 2005] and [Zhuo et al., 2006]), myosin ([Campagnola and Loew, 2003], [Boulesteix et al., 2004], [Chu et al., 2004] [Plotnikov et al., 2006]) and tubulin (Campagnola et al., 2002) scaffolds. In contrast to multiphoton fluorescence, SHG is a second order coherent nonlinear optical process providing additional information about the microscopic architecture of tissues. The SHG light is easily filtered from fluorescence as it is generated at doubled frequency of the driving laser field (Boyd, 2003). Harmonic generation processes such as SHG and THG do not necessarily involve electronic excitations. In other words, due to the absence of energy deposition into the sample, photo bleaching effects are suppressed and structures of interest can be observed intact for a longer period of time.

CARS microscopy derives its imaging contrast from Raman-active vibrational modes ([Duncan et al., 1982] and [Zumbusch et al., 1999]) and has been used for cellular ([Cheng et al., 2002a], [Nan et al., 2003] and [Hellerer et al., 2007]) and tissue imaging ([Wang et al., 2005], [Evans et al., 2005]). Unlike SHG and CARS, for tightly focused laser beams, a THG signal is only generated when a medium is optically heterogeneous (both linearly and/or nonlinearly) within the focal volume scale ([Squier et al., 1998], [Chu et al., 2004], [Oron et al., 2004], [Débarre et al., 2006] and [Tai et al., 2006]). Recently, it has demonstrated that THG microscopy can reveal the presence of lipid bodies within thick tissue and be efficiently combined with multiphoton fluorescence and SHG microscopy (Débarre et al., 2006). Thus, based on different contrast mechanisms for the three techniques (SHG, THG, CARS), the simultaneous application of these is expected to reveal valuable and complimentary information about the tissue under investigation.

In this paper, we report multimodal nonlinear optical microscopy of thin fascia *ex vivo* tissue, a tissue sheath rich in collagen type-I molecules. By combining SHG-THG and CARS microscopy within the same imaging platform, we demonstrate that unique and important complementary information can be obtained about the overall structure and morphology of muscle fascia. The combined multimodal imaging of *ex vivo*, intact fascia reveals how collagen type I molecules are organized into complex tissue structures, from fibrils to fiber bundles, their orientation and organization as well as their organization into wider individual sheets forming the dense connective tissue structure of muscle fascia. Spectroscopic contrast due to CH₂ vibration bands is easily detectable for the collagen sheet by the CARS technique. We show that the forward signal CARS (F-CARS) image provides structural information that is morphologically similar to backward signal SHG (B-SHG). The THG signal is only seen at the boundaries between the collagen sheets. This observation and the weak backward CARS signal lead to the conclusion that the composition of those structures is optically homogeneous on the scale of the laser wavelength. We also obtained AFM data to interpret the results of THG and CARS microscopy. The data obtained in this work provide useful information on the origin of nonlinearities within complex collagen tissue arrays.

2. Materials and methods

Tissue preparation

Fascia tissue was harvested *in situ*, under a Nikon dissecting scope (0.8 – 4x magnification) from the tibialis anterior muscle of C57/B6 mice after euthanasia. The fascia tissue was fixed for 1–2h in 4% PFA at 4°C. A 3mm × 3mm fascia piece was transferred to a No.1 coverslip (VWR International, West Chester, USA; 24mm×60mm, No.1). The thickness of fascia is naturally about 10µm. The coverslips were treated with gelatin-chromium potassium sulfate solution (Gelatin type A- Sigma®, Chromium potassium sulfate, Sigma) for optimal tissue adhesion.

Imaging system

The laser source for SHG and THG microscopy is a Ti:Sapphire oscillator (~140 fs pulse, 76 MHz repetition rate; MIRA 900-F; Coherent Inc.,) set at 900 nm wavelength. We placed an achromatic half-waveplate that was used in conjunction with a Glan-Thompson polarizer in the optical path to attenuate the power and control polarization of the laser field. The polarizer is located at the input port the microscope and is set to fix the laser polarization in p-plane at the entrance of the laser scanner.

The laser sources that are used to generate CARS images consist of a passively mode-locked Nd:YVO₄ laser (High-Q Laser GmbH) operating at 1064.2 nm (7 ps, 10W, 76 MHz) and an optical parametric oscillator (OPO) synchronously pumped by the Nd:YVO₄ laser. The OPO is temperature tuned and the intracavity doubled radiation provides a tunable output from 800–900 nm. Typically, the OPO delivers ~1W output power within the above tuning range, with pulse durations slightly shorter than the ones for the Nd:YVO₄ laser. Degenerate two-color CARS arrangement was used to obtain images. The OPO output served as the source of pump photons while the remaining fraction (~1.3W) of the Nd:YVO₄ laser was used as Stokes beam in the CARS process. We placed achromatic half-waveplates into both optical arms. Combined with the above mentioned Glan-Thompson polarizer this allowed us to independently adjust power of the two beams. The laser beams are collinearly combined with the use of a 950 nm long pass dichroic mirror (Chroma Technology, Brattleboro, VT). The longitudinal overlap was achieved by controlling the divergence of the pump laser with a 1:1 telescope. The temporal overlap between the pump and the Stokes pulses was controlled using an optical delay line in the pump beam optical arm. The temporal and spatial overlaps of the pump and Stokes laser pulses were optimized for observing the CARS signal from dodecane that is spread on a microscope glass slide.

The laser beams are guided with two mirrors into the NIR port of the inverted Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc., Thornwood, USA). Laser scanning is performed using the LSM 510 module from Carl Zeiss MicroImaging, Inc. Finally, the laser beams were focused into the sample with a 63X water immersion objective (C-apochromat, NA=1.2, Carl Zeiss). We imaged with a 1x physiological salt solution (PBS) added to the fascia sample to prevent drying artifacts. Forward and backward direction imaging of thin fascia samples were both enabled.

For the backward signal detection mode, the signal is deflected towards the photomultiplier tube (PMT) detector using a dichroic mirror (long pass 700 nm, Chroma Technology Corp). For the forward detection mode, the SHG-THG-CARS signals were collected using a custom-built condenser (NA=0.8) whose transmission is extended into the UV wavelength region. In addition, all the optical components that are needed to collect light in forward direction have been replaced with identical parts made from fluorite glass (Edmunds Optics) to warrant maximum optical transmission for 300 nm light. For SHG imaging, the signal is filtered with a narrow bandpass filter centered at 445 nm and a 2 mm thick colored glass filter (Schott glass, BG39). For THG, we used a bandpass filter centered at 300 nm. The CARS signal was filtered with a bandpass filter centered at 660 nm combined with a short pass 750 nm filter. All the bandpass filters were from Chroma Technology Inc. The images have been acquired and processed using software that is provided with the microscope's data acquisition system.

The AFM images were taken on an Asylum MFP-3D setup (Asylum Research 6310 Hollister Ave Santa Barbara, CA 93111). The scanning was performed in contact mode using the Olympus OMCL-TR800PSA-1 tip from SiN (silicon nitride) material. The cantilever spring constant is 0.57 N/m and its resonant frequency is 73 kHz.

3. Results and discussion

Muscle fascia as a member of the connective tissue types, is a dense fibrous connective tissue made from primarily collagen type I molecules. Muscle fascia is a three-dimensional sheet structure enwrapping muscle bundles. It maintains structural integrity, and is involved in intercellular communication. Using SHG, THG and CARS microscopy techniques, muscle fascia tissues can be imaged conveniently, and provide structural information about the level of fibrillar organization, without necessitating external fluorochromes.

Imaging of ex vivo muscle fascia by detecting forward signal

Forward SHG-THG-CARS images from the muscle fascia tissue (thickness $\sim 10\mu\text{m}$) explanted from the tibialis anterior muscle of C57/B6 mice are presented in Figure 1. Special care has been taken to provide spatial overlap between the three types of images. For the forward generated SHG-THG-CARS images, striking differences are observed. Detected features from the forward SHG image confirm the histological architecture and structure of muscle fascia. Namely, parallel collagen type-I fibrils and fiber bundles with diameters on the sub-micron to micron spatial range are detected. The CARS image taken at Raman shift frequency $\Delta\omega=2845\text{ cm}^{-1}$ does not reveal fibers or fiber bundles. Instead, the collagen architecture was found to be nearly uniform revealing only collagen sheets, 10 – 20 microns wide (Loetzke, 1956). For the THG image, we only observe a signal at interfaces, between the collagen sheets, where the optical properties are changing within the scale of the focal volume ($1\mu\text{m}^3$).

Compared to the bulk areas of the sheets, the boundary areas between them have different optical properties, such as refractive index and third order non-linear susceptibility $\chi^{(3)}$. Different density and composition of the matrix material within the spaces, between the sheets, are confirmed by a weaker CARS signal and absence of a SHG signal. Absence of a SHG signal in between the sheets indicates lack of collagen type I molecules. The material between the sheets is mostly PBS solution. This is supported by the fact that we observe a CARS signal intensity typical for that solution. Absence of a THG signal within the collagen sheets suggests that they are of mostly optically homogeneous bulk media within the scale of the optical wavelength. The homogeneous quality pertains to both the linear and nonlinear optical property given that the THG signal depends on changes in linear refractive and third order nonlinear susceptibility indices within the focal volume (Ward and New, 1969).

In our experiments, the laser polarization has been fixed to be parallel with the axis of the collagen fibril. This particular orientation provides maximum SHG signal (Williams et al., 2005) while for THG and CARS microscopy, we have not observed any significant dependence of the signal.

Overall, the THG signal was weak for our experimental conditions. However, we detected boundaries between the collagen sheets with good contrast, which is one of the demonstrations of the usefulness of this technique. The THG signal can be increased using more intense laser sources that emit at longer wavelengths (1200 nm – 1450 nm). A higher intensity will not introduce photodamage to the sample as the corresponding threshold increases at these wavelengths ([Débarre et al., 2006] and [Tai et al., 2006]). In addition, the light collection and PMT sensitivity are more efficient in the visible spectral range.

CARS microscopy derives its imaging contrast from Raman-active vibrational modes. Within the OPO wavelength tuning range (804 nm – 821 nm), the frequency difference between the pump (ω_p) and Stokes lasers (ω_s) spans the range of 2785 cm^{-1} – 3040 cm^{-1} . This range is specific for CH_2 vibrations ([Cheng et al., 2002a], [Nan et al., 2003], [Hellerer et al., 2007], [Wang et al., 2005] and [Evans et al., 2005]) where the CARS signal is easily detected due to the high density of the modes. The primary sequence of collagen contains amino acid repeats

with a relatively high content of CH₂, such as Proline and Hydroxyproline (see also below). The F-CARS image has been generated at a Raman shift frequency $\Delta\omega = 2845 \text{ cm}^{-1}$ as shown in Figure 1.

Figure 2(a–b) presents F-CARS images of fascia for two distinct $\Delta\omega$ values corresponding to the CH₂ vibrational resonance ($\Delta\omega = 2845 \text{ cm}^{-1}$) and for the off-resonance condition ($\Delta\omega = 2990 \text{ cm}^{-1}$). Figure 2(c) shows the corresponding image of forward signal SHG (F-SHG). The left half of the image represents the buffer solution area in which the sample was held to prevent drying artifacts. The other half of the images shows the tissue area. The contrast in CARS images strongly depends on $\Delta\omega$ thus indicating CH₂ vibrational contrast.

In stark contrast to F-SHG, the F-CARS signal is nearly uniform within the collagen sheets indicating that the physical density that relates to resonant $\chi^{(3)}$ of the C-H rich biomolecular structures is uniform (Oudar and Shen, 1980). At the same time, the signal intensity for the SHG images is spatially modulated across the sheet. Collagen fibrils and their arrangement within the fascia matrix sheets are highly complex as they are more than just straight rods ([Gale et al., 1995], [Holmes et al., 2001] and [Canty and Kadl, 2002]). The SHG signal intensity depends on both the density of $\chi^{(2)}$ sub-structures (collagen type I molecules) as well as their macroscopic arrangement that leads to higher non-centrosymmetry. Within the focal volume, a random spatial distribution of a large number of collagen type-I structures that have the same $\chi^{(2)}$ will suppress the SHG signal due to the destructive interference effect (similar to collagen type I powder (Theodossiou et al., 2006)). Therefore, the strong modulation of the SHG signal across the imaging area of fascia can be due to a different density of collagen type I molecules as well as the degree of noncentrosymmetry within the focal volume.

The CARS signal that originates from the nonlinear interaction of the pump and Stokes lasers in the buffer medium (see Figure 2) is non-resonant. This signal can be used for normalization while measuring the CARS spectra. Figure 3 presents the normalized CARS spectrum from $2785 \text{ cm}^{-1} - 3040 \text{ cm}^{-1}$ vibrational frequency region showing two characteristic peaks. The first peak is located at $\Delta\omega = 2860 \text{ cm}^{-1}$ and can be attributed to the symmetric aliphatic-CH vibrational mode while the second one at $\Delta\omega = 2925 \text{ cm}^{-1}$ is attributed to the asymmetric aliphatic-CH vibration mode. The dip in the spectrum at $\Delta\omega = 2990 \text{ cm}^{-1}$ is due to the destructive interference between the electric field amplitudes corresponding to real and imaginary components of $\chi_{\text{vib}}^{(3)}$ ([Potma and Xie, 2003] and [Müller and Zumbusch, 2007]). Thus, our results demonstrate that CARS microscopy has the ability to image collagen arrays with CH₂ spectroscopic contrast. The contrast and the shape of the spectrum are due to collagen proteins that are composed of Glycine and Proline or Glycine and Hydroxyproline amino acid repeats. Proline and Hydroxyproline each contain a pyrrole ring containing several CH₂ bonds in its ring backbone. Those eventually contribute to a strong resonant CARS signal.

Backward imaging of ex vivo muscle fascia

For thick and *in vivo* tissue imaging, backscattering of forward generated light can contribute significantly to the signal detected in backward direction. This feature has been demonstrated experimentally for SHG (Légaré et al., 2007), THG (Débarre et al., 2006) and CARS microscopy techniques (Evans et al., 2005). Using natively thin fascia tissue with a thickness near 10 microns, which is much less than the characteristic main free path for the laser photons (Chan et al., 2005) used in the experiments, we can neglect backscattering of the forward light and precisely characterize backward generated light ([Légaré et al., 2007] and [Pfeffer et al., 2007]). In the case of THG imaging, we were not able to collect the backward generated light due to the constraints of the setup configuration discussed in section 2.2. For SHG and CARS microscopy, we collected the signals in backward direction by adding a thick column of PBS onto the thin section of fascia as described recently (Légaré et al., 2007). This procedure assures

that the backward signal is not contaminated with the forward signal as a result of light reflection at the PBS-air interface.

Under the described experimental conditions, the SHG and CARS images have been captured in backward as well as in the forward directions. With a thick column of PBS on top of the thin sample, we cannot use the 0.8 NA custom built condenser due to the small working distance (see section 2.2). For the forward images in Figure 4, we used a 0.5 NA condenser from Zeiss (26 mm of working distance). F-CARS and B-CARS images are presented in Figure 4(a,b). The B-CARS signal within the sheets is three-orders of magnitude weaker than the F-CARS signal. Initially, we were concerned that the B-CARS signal within the sheets arises from Fresnel reflection. However, by comparing B-CARS and F-CARS images, one can see that the important features observed in the forward direction are absent in backward. If the backward signal will be the result of Fresnel reflection, those features will be observed. In addition, as reported by ([Volkmer et al., 2001]) and [Cheng et al., 2001b]) for cellular arrays, we show that B-CARS microscopy highlights sub-micron structures and interfaces due to incomplete destructive interference.

F-SHG and B-SHG images, presented in Figure 4(c,d), have been taken at the same location as the CARS images. The B-SHG signal is about five times weaker than the forward one. The ratio of forward over backward signal has been measured using the same experimental procedure as was described by (Williams et al., 2005). The observed features differ strongly from those of the F-SHG image (Figure 1(a)). Heterogeneous sub-micron features dominate the B-SHG image, clearly marking the sheet nature of the collagen organization of fascia. The B-SHG image shows many similarities with F-CARS image as the two highlight the sheet-like architecture of the collagen array. The observed heterogeneous sub-micron features of the backward SHG image indicate that the spatial distribution of the second order nonlinear optical susceptibility within the collagen type I architecture and the molecular arrangement within the sheets is highly complex and heterogeneous. The collagen molecules do not appear to be simply straight rods arranged in parallel straight bundles as can be assumed by considering only the F-SHG images. However, it is interesting to note that the collagen fibrils are arranged to form sheets with clearly defined boundaries. The mechanism that controls the sheet formation and width remains unclear.

We have seen in section 3.1 that the THG signal is only detectable at the boundaries between the collagen sheets. In fact, this can be very well expected as the interface area is composed of two optically different media and is on the scale length of the focal volume. Contrary to that observation, the THG signal is absent within the collagen sheets. The structural architecture of the collagen sheets is known to be highly heterogeneous on the nanometer scale as supported by electron microscopy data ([Holmes et al., 2001] and [Meadows et al., 2000]). Therefore, we explain the absence of the THG signal by the fact that the spatial scale of the heterogeneities is much smaller than the dimension of the focal volume. Therefore, the total THG signal is suppressed and not detectable. The strong suppression of the backward CARS signal can be considered along similar arguments. In order to explain those results, we performed AFM imaging of fascia. In figure 5, we present an AFM image on a spatial scale similar to the one in Figure 4. We observed that the density of biomaterials within the collagen sheets is mostly uniform, explaining our experimental observations with THG and CARS microscopy techniques.

The observation of strong B-SHG signal (only 5 times less than the forward signal) indicates that the spatial distribution of $\chi^{(2)}$ sources within the sheet is different than the $\chi^{(1)}$ and $\chi^{(3)}$ distributions. The distribution of $\chi^{(1)}$ and $\chi^{(3)}$ is associated with molecular density as opposed to $\chi^{(2)}$ which is related to the level of noncentrosymmetry (no center of inversion). Indeed, as it was reported in the paper by (Williams et al., 2005), the SHG signal originates from the fiber

bundle shell. The characteristic diameter for the bundle ranges from a few hundred nanometers up to the micron scale which is on the same order as the dimension of the focal volume (optical wavelength).

4. Conclusion

We demonstrate a combined SHG-THG-CARS imaging approach to study fascia. Using this multimodal approach, we obtain complementary information about the collagen array, such as the overall fibrillar architecture (SHG), the boundaries between the collagen sheets (THG) and their widths (CARS). Within the sheet, we observe CH₂ spectroscopic contrast. In addition, using F-CARS measurements, we observe that the physical density of the C-H rich biomolecular structures within the collagen sheets is rather uniform. Second harmonic images reveal the fibrillar architecture of fascia and confirm a rather high degree of heterogeneity of $\chi^{(2)}$ within the image plane.

In the backward direction, only SHG and CARS images have been characterized. The level of B-SHG signal is on the order of the one observed for the forward direction, as opposed to CARS where the backward signal is three-orders of magnitude weaker than in forward. This demonstrates that the spatial distribution of $\chi^{(2)}$ in the collagen sheet is heterogeneous (Mertz and Moreaux, 2001) and is different from the $\chi^{(1)}$ and $\chi^{(3)}$ distributions. The heterogeneous features of sub-micron size observed in Figure 4 are intrinsically linked to the complex spatial distribution or modulation of $\chi^{(2)}$ within the collagen array. In order to characterize this complex nanoscale arrangement, imaging approaches sensitive to $\chi^{(2)}$ but with higher spatial resolution, such as SHG-NSOM ([Shen et al., 2001] and [Schaller et al., 2003]), would be desirable.

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1. Multimodal nonlinear optical imaging of fascia. (a) forward-SHG image, (b) forward-CARS image at Raman shift frequency $\Delta\omega=2845\text{cm}^{-1}$ and (c) forward-THG image. Scanning area was $146.2\mu\text{m}^2$ (512×512 pixels).

2.
SHG-CARS imaging of fascia. Forward-CARS image at Raman shift frequency: (a) $\Delta\omega=2845\text{cm}^{-1}$ and (b) $\Delta\omega=2990\text{cm}^{-1}$. (c) Forward-SHG image. Scanning area was $73.1\mu\text{m}^2$ (512×512 pixels).

3. Normalized CARS spectrum of fascia. $\Delta\omega$ denotes the frequency difference between the pump and the Stokes lasers. S_{fascia} corresponds to the average CARS signal from fascia and $S_{\text{non-resonant}}$ is CARS signal generated in the buffer solution.

4. Forward-backward SHG-CARS imaging of fascia. Forward-CARS image at Raman shift frequency $\Delta\omega=2845\text{cm}^{-1}$. (a) Forward-CARS image, (b) Backward-CARS image, (c) Forward-SHG image, and (d) Backward-SHG image. Scanning area was $73.1\mu\text{m}^2$ (512×512 pixels).

5.
Atomic force microscopy image of fascia in contact mode. Scanning area was $90\mu\text{m}^2$ (256×256 pixels).