High resolution imaging of skeletal muscle by second harmonic generation

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Abstract

Only a limited number of highly ordered bio-molecules or macromolecular structures have been shown to exhibit second harmonic generation (SHG), two of them are myofibrils and collagen fibers, both major structures in skeletal muscle. In this study, we demonstrate that microscopy based on SHG not only allows imaging of unfixed and unstained thick pork muscle, but also results in images of comparable resolution to standard histological procedures. Second harmonic generation microscopy is a promising new technique for biological imaging with many advantages over existing one-photon fluorescence techniques. It is based on a non-linear optical process which can take place in the focal volume of a microscope using femto second pulsed laser light. While two-photon fluorescence involves the near-simultaneous absorption of two photons to excite a fluorophore, followed by relaxation and non-coherent emission, SHG is a nearly instantaneous process in which two photons are converted into a single photon of twice the energy, emitted coherently. The SHG occurs when an intense laser beam passes through and interacts with a highly polarisable material with noncentrosymmetric molecular organization.

Introduction

Profound knowledge about the mechanism of the interaction of molecules in relation to microstructures is of prime importance in order to produce meat and meat products of requested structure, safety and overall quality. Therefore classical microscopy has always been an important tool in the field of Meat Science. In recent years, fluorescence microscopy in combination with Confocal Scanning Laser Microscopy has becoming the method of choice for visualization of minor molecules (Brüggemann and Lawson, 2005). The use of light, rather than traditional histological sectioning methods provides a minimally invasive method of visualising food structures and can be used to study static phenomena as well as dynamic processes. However, most of the excitation schemes require that the specimen contain either intrinsic or extrinsic fluorescent probes in order to produce contrast. Recently, more focus has been on the development of non- linear methods, which create significant contrast based on the chemistry, the organisation and/ or the orientation of nanostructures in specimen.

Second harmonic generation is a nonlinear optical process, in which photons interacting with a nonlinear material are effectively "combined" to form new photons with twice the energy, and half the wavelength of the initial photons. For example using a near infrared excitation laser at a wavelength of 800 nm will result in a blue SH signal at 400 nm. It was first demonstrated by Franken et al. at the University of Michigan in 1961 and was only made possible by the invention of laser technology in 1960, which created the required high intensity monochromatic light. In the meantime, it has been shown that not only crystals but also some biological specimen like skeletal muscle and collagen exhibit strong SHG.

Material and methods

Samples of pork *M. biceps femoris* have been taken at 24 h post mortem, snap frozen in liquid nitrogen and stored at -80 ºC until further processing. 30 µm thick section have been cut using a cryo-microtome (Reichard-Jung, Germany). Samples were rehydrated in 0.1 M posphate buffered saline and mounted in DABCO. The images were made using a custom built multiphoton excitation microscope. The microscope is build around an Olympus IX70, which was specially adapted for laser scanning, confocal, two photon and, second harmonic imaging. The microscope is a coupled with a Spectra Physics Mai-Tai (wideband Mai-Tai, 720-990 nm) Ti:sapphire femtosecond laser for the two photon excitation experiments. The excitation wavelength was 800nm and was circularly polarized. An Olympus 60X water objective NA1.2 was used. The transmitted light was collected by a 20X Olympus air objective and passed through a multiphoton emitter (Chroma ET680SPUV) and a 3 mm thick BG12 filter. The back scattered light was passed through a multiphoton emitter (Chroma ET680sp) and a 400±10 nm band pass filter.

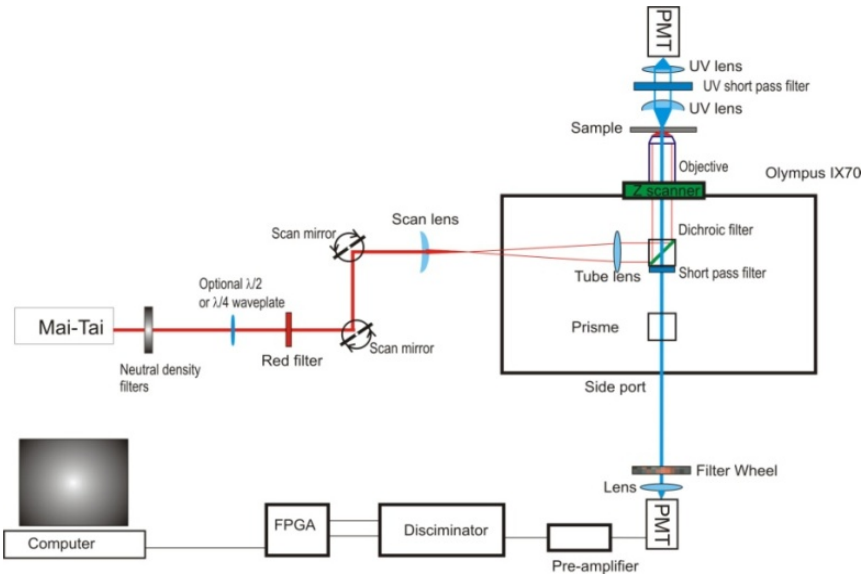

Figure 1. Schematic representation of the custom build microscope set up.

Results and discussion

In pork muscle strong SHG signals can be recorded using a femtosecond laser source at 800 nm and recording the emission at 400 nm (±10nm). The sarcomeres of skeletal muscle and collagen fibrils are clearly visible at high resolution as demonstrated in Figure 2. In general it is known, that the SHG wave is primarily emitted in the forward direction, but in thicker, turbid and highly scattering tissue also backscatterd SHG have been observed (Légaré et al., 2007).

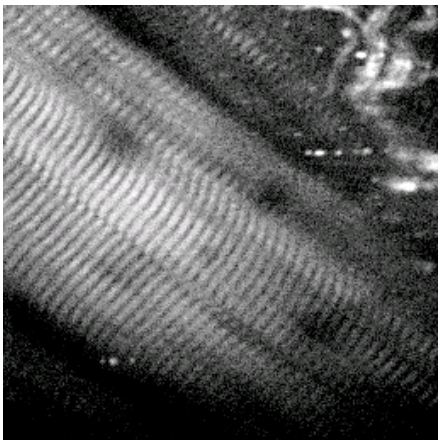

Figure 2. Image showing the sarcomeric pattern of two neighboring myofibers and collagen fibers in pork *M. biceps femoris* based on SHG emission at 400 nm. Image dimension 110 µm x 110 µm.

In Figure 3, a direct comparison of the influence of the directionality of the SHG propagation is shown. Forward transmitted SHG result in stronger signals from the myofibers (a), while collagen fibrils in the endomysium become much better visible in backscattered SHG (b).

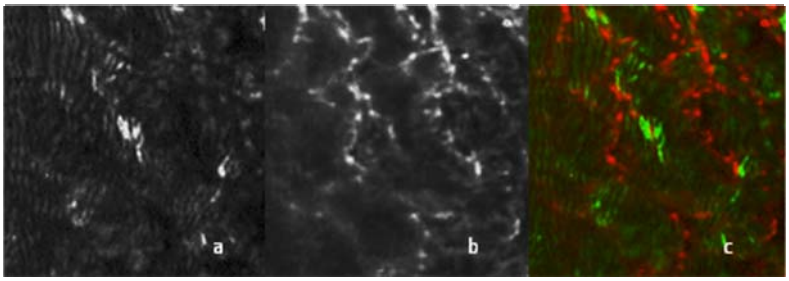

Figure 3. Images of pork muscle fibers generated by forward propagated SHG (a) and backscatted SHG (b). Merged image (c) of the two channels (a) green and (b) red. Image size 18.7µm x 18.7µm.

A false coloured merged image (c) of the two channels demonstrates that there is no overlap in the signal generated between the two detection modes. These results are in accordance with Légaré et al. (2007), which also found a reduced backscattering for skeletal muscle tissue in comparison to tendons. The authors explain this phenomenon by differences in the orientation of the muscle striation to the incident laser propagation axis. However, a direct comparison of forward and backscattered SHG in muscle perimysial collagenous structures also resulted in the visualisation of different structures.

Only a limited influence of the polarization angle of the excitation beam on backscattered SHG signal distribution or intensity was found for collagen fibrils in the endomysium of the muscle. As shown in Figure 4, no difference could be perceived in the macro structures displayed. All images show a capillary connecting an arteriole to a venule. Yet peak intensities within the collagenous structures composing the vasculare wall variied by a factor of two. This result is surprising, because in tendon a much larger effect of the polarization angle on the image formation and signal intensity has been demonstrated by William et al. (2005).

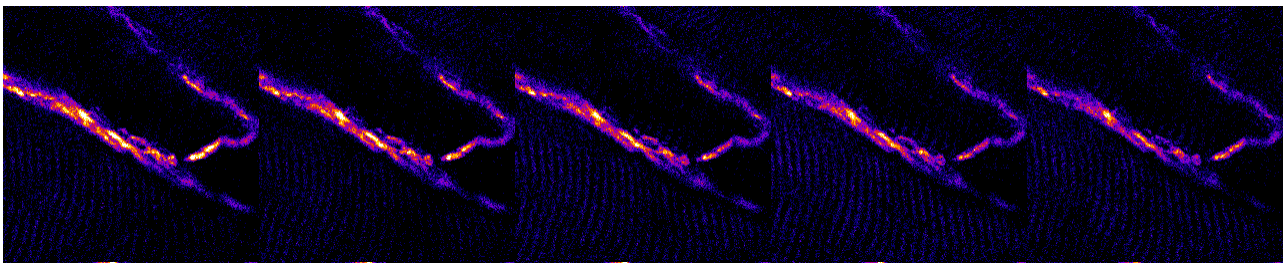

Figure 4. Backscattered SHG images of *M. biceps femoris* showing a capillary connecting a muscle arteriole to a venule in the endomysium . The images are false coloured according to the intensity of the signal. In each image the polarization angle of the excitation beam is clockwise rotated by 20º - ranging from -40º (left) to 40º (right) . Image size 37.4µm x 37.4µm .

Reference

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