Selective Harmonic Optical Microscopy

Differentiating Tissue Types with Second Harmonic Generation

SHG is an important contrast mechanism in optical examination for thick biological tissues. Fibrous proteins, such as myosin and collagen, exhibit biophotonic crystal nature and are dominant SHG harmonophores in vertebrates. Local molecule arrangements strongly affect SHG polarization behavior. Here we demonstrate to distinguish myosin-based muscle fibers from intertwined collagenous perimysium through SHG polarization selection, without complicated staining or sample/image processing required.

Second Harmonic Generation -Microscopy (SHGM)

In recent years, SHGM has emerged as an important microscopic modality. Due to its nonlinear nature, SHGM exhibits an optical sectioning power, making three-dimensional observation feasible inside a thick tissue. Since it is allowed only in noncentrosymmetric media, SHG has been used to probe photonic crystal-like structures in biological tissues, such as cellulose fibril in cell walls, amylopectin/amylose crystallized layers in starch granules, collagen fibrils in connective tissues, actomyosin lattice in muscle fibers, and orderly-arranged microtubule in mitotic spindles [1]. In contrast to laser-induced fluorescence, SHG deposits no energy to the matters with which it interacts due to its energy conservation characteristic. For most studies, intrinsic SHG signals provide the contrast and thus no staining is required. The energy-conservation and label-free characteristics enable optical noninvasive investigations, which is desirable for in vivo biological microscopic applications. Combined with a near-infrared laser source, the deep-penetration capability makes this imaging modality suitable for tissue characterization.

Different from the incoherent mechanism involved in a fluorescence process, coherent SHG exhibits a specific polarization relationship. The polarization dependence of the emitted SHG radiation is strongly affected by the arrangements and structures of the constructing molecules in biological tissues [2]. This provides the basis for selective imaging on different molecules through appropriate polarization manipulation [3].
**Methods**

To demonstrate polarization based selective imaging, a mouse skeletal muscle tissue was chosen as the model system.

The tissue consists of striated muscle fibers held together by type I collagen based perimysium, which is the connective tissue that ensheathes a muscle fascicle. Though significant morphological differences exist between connective tissue fibers and muscle fibers, it is difficult to differentiate them inside a thick tissue without specific staining under a conventional microscope. Both perimysial collagen and muscle fibers are known to exhibit strong SHG emission, but there has been no report on distinguishing their SHG yet. Here we demonstrate that through proper polarization manipulation, the coherent SHG from intertwined muscle and collagen fibers can be effectively separated, allowing high contrast selective imaging in thick bio-tissues with sub-μm resolution.

Our home-built laser scanning polarization SHG microscope has been described previously [2]. A Cr:forsterite laser operating at 1230 nm was used as the light source to provide high penetration into biological tissues. High-speed scanning was accomplished through galvanometric mirrors built in the Olympus FV300 scanner. The incident laser polarization was manipulated by a half-wave plate. An analyzer was inserted in front of the photomultiplier tube (PMT) to determine the polarization state of the emitted SHG radiation. Mouse skeletal muscle tissues were dissected from the leg of a 1-year-old mouse and preserved in formalin.

**Results**

We first determine the SHG polarization anisotropies of collagen and muscle fibers respectively, as shown in figure 1a with intertwined perimysial collagen fibers around a muscle fiber background. Due to the distinct coil formations in collagen and in myosin, distinctive polarization characteristics from the two structural proteins are expected. Two regions, which respectively contain only muscle...
(rectangular area) and collagen (elliptical area) fibers, are selected for quantitative analysis. The type I collagen fibers and myofibers in the selected region are parallel to each other. Here the image contrast is defined as contrast = (SHGcollagen - SHGmuscle) / SHGmuscle in the muscle-collagen network. SHG anisotropies without an analyzer are shown in figure 1b, and are in good agreement with previous studies [4, 5]. From the anisotropies, the contrast is expected to be greatly enhanced when the excitation polarization is parallel to the longitudinal axes of both muscle and collagen fibers.

The emitted SHG polarization from the selected collagen and muscle fibers are analyzed in figures 1c and 1d, with horizontal and vertical laser polarization, respectively. The resulting collagen contrast enhancement is summarized in table I. Substantial contrast enhancement over two orders of magnitude is observed with suitable polarization arrangement. Such contrast enhancement and selective imaging of collagen fibers over muscle fibers are manifested in figure 2, with four possible polarization arrangements. When both laser polarization and analyzer orientation are horizontal (perpendicular to the muscle fibers, figure 2a), strong SHG from the muscle fibers is observed. It is difficult to distinguish perimysial collagen fibers in this case. In figure 2b, when the laser polarization is kept horizontal but the analyzer is switched to vertical, SHG from muscle is filtered out. Nevertheless, some muscular SHG still transmits through the vertical analyzer and the intensity of this residual SHG is comparable to the collagenous SHG. Therefore, selectivity cannot be accomplished simply by inserting a gating analyzer in front of the detector.

With vertical laser polarization (parallel to the muscle fibers), SHG from the muscle fibers is strongly suppressed, so that SHG from the perimysial collagen stands out, as shown in figures 2c and 2d. Some striated patterns are discernible in the collagen fibers. Because we collect the forward propagating SHG, after it passes through the underlying thick muscle fibers with alternating A- and I-bands, a banding pattern is left on the collagen SHG images.

When muscle and collagen fibers are oriented in the same direction, better contrast is expected with laser polarization parallel to them. The higher the contrast, the easier it is to resolve collagen fiber distribution on a massive muscle background. Since collagen fibers form a matrix in perimysium, it is important to determine the contrast when collagen fibers are not parallel to the muscle fibers. From figure 3, the contrast of a horizontal collagen fiber (ellipse in fig. 3) over the background vertical muscle fibers (rectangle in fig. 3) is enhanced from (509-2079) / 2079 = -0.78 in figure 3a to (3340-20) / 20 ~170 in figure 3b. This contrast enhancement is similar to what we expected from figure 1.

One more thing notable from figure 3 is that striated muscle fibers tend to organize in a longitudinally parallel array in most skeletal muscle tissues, so it is possible to
selectively visualize the collagen fiber distribution in an extended area without the interference of the strong SHG from muscle fibers. With appropriate laser polarization and analyzer orientation, the overall contrast of perimysial collagen matrix is greatly enhanced over a thick muscular tissue. To obtain statistically significant results, we examined over 10 muscle tissues with endogenous collagen matrix. The average contrast when the laser polarization is perpendicular to the muscle fibers is $0.17 \pm 0.3$. When the laser polarization is parallel to the muscle fibers, the average contrast increases dramatically to $267 \pm 66$. The mean enhancement factor is $580 \pm 180$. Therefore, our polarization-based technique provides more than 2 orders of magnitude of contrast enhancement on collagen over muscle tissue in SHG microscopy.

**Conclusion**

We have demonstrated polarization based selective SHG imaging in vertebrate tissues. Due to its strong dependency on symmetry, SHG is sensitive to local molecule assembly configuration and orientation. Myosin in muscle fibers and collagen in connective tissues exemplify biophotonic-crystallized structures in vertebrate tissues. Exploiting the unique polarization dependencies of these SHG-active molecules, detailed collagen fiber distributions can be highlighted with greatly improved contrast over a muscle tissue background. Our technique provides a general tool for examinations of muscle/connective tissues, which occupy more than 30% in human body mass. The potential applicability includes the study of the collagen dynamics during muscle development in vertebrates as well as screening and monitoring of collagen-related muscular diseases, such as myocardial fibrosis. This polarization based technique is ready to be extended to other structural proteins under a SHG microscope.

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