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Absolute polarity determination of teeth cementum by phase sensitive second harmonic generation microscopy

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ABSTRACT

The absolute sign of local polarity in relation to the biological growth direction has been investigated for teeth cementum using phase sensitive second harmonic generation microscopy (PS-SHGM) and a crystal of 2-cyclooctylamino-5-nitropyridine (COANP) as a nonlinear optic (NLO) reference material. A second harmonic generation (SHG) response was found in two directions of cementum: radial (acellular extrinsic fibers that are oriented more or less perpendicular to the root surface) and circumferential (cellular intrinsic fibers that are oriented more or less parallel to the surface). A mono-polar state was demonstrated for acellular extrinsic cementum. However, along the different parts of cementum in circumferential direction, two corresponding domains were observed featuring an opposite sign of polarity indicative for a bi-polar microscopic state of cellular intrinsic cementum. The phase information showed that the orientation of radial collagen fibrils of cementum is regularly organized with the donor (D) groups pointing to the surface. Circumferential collagen molecules feature orientational disorder and are oriented up and down in random manner showing acceptor or donor groups at the surface of cementum. Considering that the cementum continues to grow in thickness throughout life, we can conclude that the cementum is growing circumferentially in two opposite directions and radially in one direction. A Markov chain type model for polarity formation in the direction of growth predicts D-groups preferably appearing at the fiber front.

1. Introduction

Optical microscopy plays a central role in biology to visualize a wide variety of cells and tissues, and can reveal much of the morphology, molecular order, heterogeneity of fiber orientation and nonlinear optical properties in collagen. Research on fiber orientation is important for clinical applications and also for industrial and cosmetological uses.

Collagen is the predominant structural protein in most biological tissues. Modification of the structure of collagen molecules and their aggregates (microfibrils, fibrils, fibers and bundles) are associated with various physiological processes, such as wound healing, skin cancer, diabetes, collagen disease, burn and osteoarthritis (Han et al., 2005; Yeh et al., 2005; Georgakoudi et al., 2002). Various methods that reveal the structure and the orientation of collagen have been described: electron microscopy (Eyden and Tzaphlidou, 2001), X-ray diffraction (James et al., 1991), microwave method (Osaki, 1999), mechanical examination (Melis et al., 2001, 2002), biochemical and histological analysis (Osman et al., 2013). However, these methods are invasive and/or destructive. In view of a preliminary diagnostic to examine whether tissue biopsy is necessary or not, a noninvasive and non-destructive methods are needed, such as optical probe methods based on optical nonlinear effects in biological tissue. Collagen fibers are the major source for a second harmonic generation (SHG) signal because they show a non-centrosymmetric triple helical structure and a large second-order nonlinear susceptibility.

The collagen SHG response can give direct information about the spatial collagen organization without any gating technique. SHG is known to provide information about the spatial dependence of the distribution of orientation of the fibrils which is not easily obtained by other means (Stoller et al., 2002). SHG light provides deep penetration power without photobleaching, phototoxicity or additional staining. Therefore, SHG is very promising as a sensitive diagnostic technique in tissue morphology and physiology studies (Chen et al., 2012). The intensity of the SHG signal is highly

Abbreviations: PS-SHGM, phase sensitive second harmonic generation microscopy; SHG, second harmonic generation; COANP, 2-cyclooctylamino-5-nitropyridine.

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dependent on the chirality of the collagen fibrils (Lee et al., 2013), that is known to change in specific pathological conditions. Several research groups have reported investigations of second order nonlinear processes in various biological systems and tissue engineering research. Mertz and Moreaux (2001) used SHG to investigate membranes and to measure inter-membrane separation. It was used to characterize membrane potentials by Campagnola et al. (1999) and Pons et al. (2003). Other examples of where SHG has been used to investigate biological samples are tubulin, actin and myosin complexes, chloroplasts, light harvesting chlorophyll a/b pigment-protein complexes of photosystem II, and the formation of polysaccharides such as starch.

Few quantitative microscopic methods such as light scattering (de Vries et al., 2000; Ferdman and Yannas, 1993), scanning electron microscopy (Ambekar et al., 2012; Parry and Craig, 1977) and SHG (Stoller et al., 2003) have been established to analyze the mean orientation of collagen in tissues and also to determinate the orientation of individual collagen fibers (Noorlander et al., 2002; Hovhannisyan et al., 2012). Based on the *reflection-type* polarization measurements of molecular SHG light, *collagen fiber orientations are different in several human tissues* (Yasui et al., 2004). Previous electron microscopy studies indicated that collagen fibrils are *oriented up and down in a random manner*, whereas sum-frequency generation experiments (Freund et al., 1986) suggested that there are *regions of fibrils oriented in the same direction.* Individual fibrils can have opposite polar axis orientations and are arranged into nano-domains having the same polar orientation according to piezoresponse force microscopy (Harnagea et al., 2010). A polarity reversal is present in some of isolated collagen fibrils (Holmes et al., 1994), thereby the transition region is not restricted to a central location in a fibril.

Freund et al. (1986) studied the polarity of connective tissue in a wet rat-tail tendon by using *transmission* SHG microscopy. Their results revealed a variety in SHG signal strength due to the degree of parallel and antiparallel orientation of neighboring collagen fibrils. Parallel orientation of neighboring collagen fibrils contributes coherently to the SHG signal, whereas the SHG light from antiparallel fibrils interferes destructively. Stoller et al. found that the diameter of collagen fibrils is probably in the order of 1 micrometer or less in regions where they are parallel (Stoller et al., 2003).

Various mathematical and computational methods to model tissue growth to understand the orientation of the collagen fiber in biological tissue have been reported (Han et al., 2013; Ehmke et al., 2014; Schriefl et al., 2012). The relation between tissue polarity and growth in the generation of different shapes was investigated through a series of computational models (Kennaway et al., 2011). Experimentally, Foolen et al. studies showed that collagen fiber directions in the perichondrium and periosteum are aligned with a preferential direction along the main bone growth (Foolen et al., 2008). However, the collagen fibers become comparatively more organized as the bone develops (Ambekar et al., 2012).

Polarity formation in natural tissues is a key issue: It plays an essential role in tissue formation, function and repair. Non-destructive methods revealing *spatial polarity distributions* at different levels of resolution are thus of interest. In contrast to most of the methods reviewed above (except scanning piezoelectric microscopy (Gruverman et al., 2007)), phase-sensitive second-harmonic generation microscopy (PS-SHGM) can reveal polar tissue structures (Rechsteiner et al., 2000). This analytical method can provide *the absolute sign of local polarity in relation to the biological growth direction*, i.e. the fibril elongation process. There is evidence that failure in proper absolute polarity of tissues can be the cause for diseases.

A Markov chain analysis using biochemical data on fibril elongation has provided a first mechanistic explanation for the occurrence of *polar tissue* in nature (Hulliger, 2002). The theoretical model also explained why tissues extending in opposite directions are showing the characteristic of a bi-polar state. However, no information regarding fiber orientation with respect to *the absolute polarity* has been demonstrated experimentally so far.

A major problem in biology still is to understand how complex tissues shape along their growth. In many cases this process involves preferential extension along particular orientations raising the question of how these orientations are specified: (i) do the collagen fibers all have the same direction or (ii) does the sign of polarity vary from fibril to fibril. Here, we studied the polarity of collagen bundles in *cementum* using PS-SHGM in order to obtain a basic understanding of the polar properties of biological tissue. An essential aim is to determinate *the absolute orientation of the local average polarization in relation to the main biological growth direction*. Knowledge on the absolute polarity distribution in tissues will be a major step forward, because it can provide complementary information on the degree of organizational disorder of the fibril structure, as many diseases are characterized by an abnormally organized or defect fibril assemblies of collagen.

2. Materials and methods

2.1. Sample preparation

Human teeth that are requiring extractions as a part of dental treatment were collected. They were cut in thin samples (ground to a thickness of about 80–100 μm) using a microtome and a diamond knife for getting ultra-sections.

2.2. Second harmonic generation (SHG) measurements

The second harmonic generation (SHG) measurements were performed using a Q-switched Nd:YAG Laser (Surelite I-10, Continuum) providing a repetition rate of 10 Hz with a pulse width of 20–25 ns and a pulse energy of ~25 mJ (1064 nm, pulse intensity 10 MW/cm^{-2}, beam diameter of 4 mm). A Leica polarizing microscope (DM RXP, Leitz) and T-scope microscope used in transmission mode, coupled to a high definition CCD color video camera (Zelos – 02150C GV) to take color pictures. Objectives of 4×, 10× and 20× magnifications (LMPLFL Olympus) were used.

2.3. Phase-sensitive SHG measurements

The SH reference beam was generated by an angle-tuned KH_2PO_4 single crystal (KDP) placed into the fundamental beam. The phase delay between the fundamental and the SH waves from the reference beam was adjusted by rotation of an angle-tuned glass plate placed between the KDP crystal and samples. Domain contrast is achieved by using the interference effect between the second harmonic waves from a sample and those from a homogeneous single-domain reference material (KDP). Interference between second harmonic waves emerging from the reference and the sample domain converts the phase information into a contrast image (see Fig. 1). PS-SHGM relies on the fact that the sign of the effective nonlinear optical coefficient d_{eff} is different for macro-domains that are symmetry related by inversion. When adjacent domains are considered, a change in the sign of d_{eff} is related to a phase shift of π for second harmonic waves emerging from either of the domains. *Therefore, PS-SHGM allows us to distinguish domains featuring an opposite sign of polarity*. For details on this technique, see references (Rechsteiner et al., 2000; Kluge et al., 2002).