Polarization-resolved four-wave mixing microscopy for structural imaging in thick tissues

Fabiana Munhoz, Hervé Rigneault, and Sophie Brasselet*

Institut Fresnel, MSAIC, CNRS UMR 7249, Aix-Marseille Université, Ecole Centrale Marseille, Domaine Universitaire de Saint Jérôme, F-13013 Marseille, France
*Corresponding author: sophie.brasselet@fresnel.fr

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We present a polarimetric analysis of the four-wave mixing (FWM) signal emitted by thick tissues, in order to extract structural information on molecular order and orientation. A careful analysis of the polarization distortions introduced by the birefringence of the sample is conducted for the proper interpretation of the results. FWM, compared to other well-known nonlinear optical methods such as second-harmonic generation, gives access to additional information on the symmetry of the molecular distribution. Furthermore, it brings the advantage of being generated in any kind of sample, even when centrosymmetry is present. The model developed here is applied to thick rat-tail tendon samples, composed essentially of collagen fibers. We show that, once the birefringence of the sample is completely characterized, it is possible to retrieve the even-order components of the molecular orientational distribution up to the fourth order of symmetry. © 2012 Optical Society of America

1. INTRODUCTION

Recent developments in polarization-resolved nonlinear microscopy have demonstrated a great potential in the study of orientational organization in ordered materials, such as crystals and biological molecular ensembles. In particular, several works have reported the use of second-harmonic generation (SHG) to retrieve quantitative order information in noncentrosymmetric media, such as molecular surfaces and monolayers [1,2], nanometric size crystals [3], collagen samples [4–8], and tissues [2–13]. In the case of collagenous tissues, probing the molecular organization can be of great interest in early medical diagnostics, because changes in the fibrillar structure of collagen may indicate different disease states. Quantitative characterization of the collagen fiber’s orientational order in tissues at the molecular scale has been reported using polarization-resolved SHG (PSHG) [5,6,8,14–18]. Polarization-resolved nonlinear microscopy allows retrieval of individual microscopic susceptibility tensor components, which can be related to valuable information on the molecular order in complex assemblies [19]. However, PSHG exhibits some limitations because, as a second-order nonlinear process, only odd orders of symmetry of the molecular orientational distribution up to the third order can be determined [19–21].

Contrary to SHG, which requires a noncentrosymmetric medium in order to take place, four-wave mixing (FWM) microscopy can be generated in any sample structure, with arbitrary organization. Because of favorable phase-matching conditions [22–25], FWM is efficient in biological tissues, as illustrated in recent works on epithelial lipids imaging in C. elegans worms [24,25], or for cytoplasmic cells imaging in zebrafish embryos during early division [25]. Contrary to third-harmonic generation (THG), another FWM process, which is interface specific [25–28], FWM is bulk sensitive and can potentially reveal structural information from macroscopic systems using a polarization-resolved analysis. In particular, polarization-resolved FWM allows one, in principle, to probe the even-order symmetries of the molecular orientational distribution, up to the fourth order [19], giving complementary information, as compared to SHG, on the microscopic organization of complex samples.

In this work, we develop the principles of polarization-resolved FWM microscopy to retrieve quantitative information on molecular order and orientation in a model system made of collagen fibers. First, we develop a general theoretical model to determine the fourth-order nonresonant susceptibility tensor of collagen, which can be applied more generally to any kind of molecular system where a degree of molecular order is present. Then, we describe the optical setup to perform polarization-resolved FWM microscopy and the method to correct from polarization distortions that can be introduced either by the optical setup or the sample itself. Indeed, biological tissues can exhibit strong scattering and birefringence, which can lead to misinterpretation of the polarimetric results, especially in thick samples [29,30]. Finally, we propose a fitting procedure to quantify orientation and symmetry orders of the molecular orientational distribution in the sample. All the studies developed here are performed in the nonresonant regime; however, they can be, in principle, extended to a vibrational resonant process, such as coherent anti-Stokes Raman scattering (CARS) to probe the orientation and symmetry of specific molecular vibrational modes [31].

2. THEORY

A. Microscopic Fourth-Order Susceptibility Tensor of a Molecular Assembly

Biomolecular organizations, such as collagen fibers, can be described as an assembly of elementary nonlinear-active molecules with a given statistical orientational distribution. This molecular angular distribution is defined by a normalized
probability distribution function \( f(\Omega) \), with \( \Omega = (\theta, \phi) \) being the spherical angles denoting the orientation of the molecular frame \((u, v, w)\) in the microscopic frame \((x, y, z)\). The use of two orientation Euler angles \((\theta, \phi)\) supposes that the molecules within the angular distribution are of uniaxial symmetry, which has been largely used in the case of collagen fibers \([5,13,16,18,32]\) and can be considered as a first approximation in an unknown sample. We assume furthermore in what follows that the molecular third-order susceptibility \( \gamma \) has only one nonvanishing component along \( w \) in the molecular frame \((u, v, w)\), \( \gamma_{\text{uvw}} \). More complex molecular susceptibility tensors can however be easily introduced in the following model. Under this molecular 1D assumption, the microscopic third-order nonlinear susceptibility tensor \( \chi^{(3)}_{ijkl} \) can be written, in the microscopic frame as

\[
\chi^{(3)}_{ijkl} = \int \gamma_{\text{uvw}}(w \cdot i)(w \cdot j)(w \cdot k)(w \cdot l)(\Omega)f(\Omega)d\Omega.
\]  

(1)

with \((w \cdot i)\) being the rotation matrix components between the microscopic and molecular frames and \( \int d\Omega = 2\pi \int_0^\pi \sin \theta d\theta d\phi \).

In this work we address the most general case, where the shape of the orientational distribution function is unknown, by decomposing it on a series of \( \Omega \)-dependent orthonormal functions \([19]\). In the context of fibrillar systems, one can furthermore assume that the molecular orientational distribution is of cylindrical symmetry; then \( f(\theta, \phi) \) does not depend on \( \phi \). The distribution function can thus be expanded in a series of the Legendre polynomials, along the same lines of previously developed models in molecular doped poled polymers \([33]\):

\[
f(\theta) = \sum_J f_J P_J(\cos \theta),
\]

(2)

where \( P_J(\cos \theta) \) is the \( J \)-order Legendre polynomial, with \( J \) representing the orders of symmetry of the orientational distribution function. The coefficients \( f_J \), called order parameters \([34]\), correspond to the weights of the function \( P_J \) in this decomposition. Increasing orders of the polynomials correspond to narrower distributions in relation to \( \theta \), together with an increasing complexity of their multipolar nature.

In the case of nonresonant FWM, a symmetric fourth-order susceptibility tensor is involved. It is thus possible to probe even components of the orientational distribution function until the fourth-order of symmetry corresponding to \( J = 4 \) \([19]\). FWM is therefore a complementary probe relative to SHG, with a sensitivity to higher details of the molecular distribution. In the context of FWM, Eq. (1) therefore becomes

\[
\chi^{(3)}_{ijkl} = \int [(w \cdot i)(w \cdot j)(w \cdot k)(w \cdot l)](\Omega)f(\Omega)d\Omega.
\]  

(3)

\[
\times \left[ 1 + f_2 \frac{3 \cos^2 \theta - 1}{2} + f_4 \frac{35 \cos^4 \theta - 30 \cos^2 \theta + 3}{8} \right] \sin \theta d\theta d\phi.
\]

where \( f_2 \) and \( f_4 \) are the second- and fourth-order parameters, respectively. These coefficients are normalized by the weight \( f_0 \) of the zeroth-order symmetry, which corresponds to the isotropic contribution of the distribution. \( f_2 \) might contain all depolarized contribution to the FWM signal, including scattering from the sample. It means that the relevant parameter in the molecular order quantification, free from unwanted isotropic contributions, is the ratio \( f_4/f_2 \). The expression of the microscopic susceptibility tensor in Eq. (3) is further normalized by \( \gamma_{\text{uvw}} \).

The decomposition of the molecular orientational distribution function in a series of the Legendre polynomials corresponds to the multipolar expansion \([29]\) of a one-dimensional structure with cylindrical symmetry. The zeroth-order term \( J = 0 \) is the monopole and corresponds to an isotropic distribution. When the coefficient \( f_2 \) is zero, then the distribution is purely hexadecapolar, with fourth-order symmetry. In the same way, when \( f_4 \) is zero, then the distribution is purely quadrupolar, with second-order symmetry \((J = 2)\). Figure 2 shows the effect of different sets of \( f_2, f_4 \) values on the theoretical molecular angular distribution function \( f(\theta) \) projected into the plane \( x-z \) as a function of \( \theta \). Note that this figure does not depict the whole distribution function, but only a truncation of its even terms up to the fourth order. When both coefficients \( f_2 \) and \( f_4 \) vanish, the...
distribution is isotropic and no direction is privileged, as expected from the zeroth-order term of the multipolar expansion. When \( f_1 = 0 \), the distribution function exhibits a two-lobe shape, that can be either along the z axis or in the plane perpendicular to it (x-y plane), depending on the sign of \( f_2 \). When \( f_2 = 0 \), the angular distribution function exhibits a four-lobe shape, which characterizes the higher multipolar order, with an orientation that also depends on the sign of \( f_4 \). Intermediate cases exhibit generally four-lobe patterns with more pronounced four lobes when \( f_4 \) is higher than \( f_2 \).

The parameters \( f_2 \) and \( f_4 \) can be directly related to a molecular orientational order information, which gives a physical picture of the way molecules are organized in the focal volume of the microscope. Indeed, typically the ratio \( f_4/f_2 \) decreases when molecules evolve from a high order (with orientations pointing along well defined directions) to a high disorder (close to isotropic distribution).

**B. From Microscopic to Macroscopic Susceptibility**

We denote \( \Omega = (\theta, \phi) \) as the orientation angles of the higher symmetry axis of the microscopic frame in the macroscopic frame (X, Y, Z), i.e., the laboratory frame where the components of the incident electrical fields are defined [Fig. 1(b)]. This high symmetry axis corresponds typically to the direction of a collagen fiber in the case of collagen-based samples. The macroscopic susceptibility tensor can be obtained by projecting the microscopic susceptibility \( \chi^{(3)}_{ijkl} \) into the macroscopic frame, according to

\[
\chi_{ijkl}^{(3)}(X,Y,Z)(\theta,\phi) = \sum_{i,j,k,l}^{(3)} \chi^{(3)}_{ijkl}(i \cdot i)(j \cdot J)(k \cdot K)(l \cdot L)(\theta,\phi),
\]

where \((i \cdot i)\) are the components of the rotation matrix between the macroscopic and microscopic frames. In what follows, we suppose that the symmetry axis of the microscopic structure (collagen fiber direction) lies in the X-Y plane; therefore, the Euler angle \( \theta_0 \) is fixed to 90°.

Finally, the FWM signal is generated from the induced third-order nonlinear polarization \( P_{FWM}^{I} \) that couples the incident electric fields with the macroscopic susceptibility tensor of the sample. In this work, we use a degenerate scheme, where two input fields have the same angular frequency \( \omega_1 = \omega_2 \) and the third one is set to the angular frequency \( \omega_3 \). In the planar wave approximation, which is found to be valid in this study [19,35], the measured intensity is proportional to the modulus square of the induced nonlinear polarization \( P_{FWM}^{I} \), at angular frequency \( \omega_0 = 2\omega_1 - \omega_3 \), and can be written as

\[
I_{I=0}(X,Y) \propto |P_{I}^{FWM}(\omega_0)|^2 = \sum_{IJKL}^{(3)} \chi_{IJKL}^{(3)}(\omega_0; \omega_1, \omega_1, -\omega_3)E_{I}(\omega_0)E_{K}(\omega_1)E_{L}^{*}(\omega_3),
\]

with \( I = X \) or \( Y \), the analysis direction along which the signal is detected, and \( E_{I}(\omega_0) \) and \( E_{I}(\omega_3) \) the components of the incident fields along the macroscopic directions \( J, K, \) and \( L \).

**3. EXPERIMENT**

**A. Optical Setup**

The incident pulse trains are delivered by two picosecond tunable mode-locked lasers (Coherent Mira 900, 76 MHz, 3 ps), pumped by a Nd:vanadate laser (Coherent Verdi). The lasers are electronically synchronized (Coherent SynchroLock System) and are externally pulse picked (APE pulse picker) to reduce their rate to 3.8 MHz. Achromatic half-wave plates mounted on step rotation motors, allow rotation of the incident linear polarizations separately for both beams. The beams are spatially recombined through a dichroic filter, injected into a commercial inverted microscope (Zeiss Axiovert 200 M), and focused in the sample through a low-numerical-aperture microscope objective (Olympus LUCPLFLN 40×, NA = 0.6), in order to avoid any contribution from the Z-polarized component of the excitation field in the experiment, while keeping a micrometric scale optical resolution [35]. The sample is placed on a piezo-electric stage (Physik Instrument) ensuring XYZ nanometric scale precision positioning. The generated signal is forwardly collected by another microscope objective (Olympus LMPFLN 50×, NA = 0.5) and is filtered by two filters (a low-pass filter to reject the incident lasers and a bandpass filter spectrally centered at the wavelength of the emitted beam). The signal is split by a broadband polarizing cube beamsplitter (Newport), and the two resulting perpendicularly polarized beams are finally detected by two avalanche photodiodes (PerkinElmer SPCM-AQR-14) used in the photon counting mode. The incident wavelengths are set to \( \lambda_1 = 724 \text{ nm} \) and \( \lambda_2 = 857 \text{ nm} \), in order to avoid any vibrational resonance from the collagen. In this work, two different schemes of polarization tuning are used: either the linear polarization of the field \( E(\omega_1) \) is fixed along the X axis and the linear polarization of the degenerate beam \( E(\omega_1) \) rotates with an angle \( \alpha_1 \) relative to X varying from 0° to 360°, or both input polarizations rotate simultaneously with an angle \( \alpha_{1,2} = \alpha_1 = \alpha_2 \) relative to X from 0° to 360°. The use of different incident polarization configurations, together with a polarized detection, is necessary in the case of birefringent samples, in order to avoid ambiguous results (see Section 4).

**B. Sample Preparation**

In this work we study collagen Type I fibers, of about 100–140 μm thick, extracted from rat tail tendons. In the sample preparation (University of Exeter, UK), adult Sprague Dawley rats were euthanized for purposes unconnected with the present research. Tails were removed and immediately snap frozen in liquid-nitrogen-cooled isopentane. At the time of use, the tissue was thawed and the tendon exposed. Individual fibers were teased out by microdissection and either examined immediately or stored frozen until required. Control Raman spectra were identical in either case, contained none of the peaks characteristic of proteoglycans, and were indistinguishable from those obtained from fibers purified by enzymatic extraction.

The sample is sandwiched between two glass coverslips glued together by a thickened double-sided tape at the edge. This procedure prevents motion of the fibers during the measurements. The volume between the two coverslips is filled in with a 0.15 M NaCl solution. Recording the FWM signal emitted by the solution allows performing of all the fine optical settings and alignments of the setup.
C. Experimental Protocol
The experimental protocol consists in the following steps. First, both incident polarizations are set parallel to each other (either $\alpha_1 = \alpha_2 = 0 \, ^\circ$ or $\alpha_1 = \alpha_2 = 90 \, ^\circ$) and two FWM images of the collagen fiber are recorded simultaneously, one polarized along the $X$ axis and the other along the $Y$ axis. The image size is typically $40 \, \mu m \times 40 \, \mu m$ (100 x 100 pixels), and the pixel dwell time is 20 ms. The average power of each of the incident beams at the focal spot position is 2 mW. The focal plane $Z$ is fixed at the bottom surface of the sample ($Z = 0 \, \mu m$, according to Fig. 3(a)). In this case, only the emitted signal is affected by the collagen fiber’s birefringence. The second step consists of choosing different ($X, Y$) positions of the acquired image in order to perform FWM polarization-resolved measurements, as described earlier in Subsection 3.A. Two experiments are performed: one for a varying $\alpha_1$ angle with $\alpha_3 = 0$ fixed, and the other for varying $\alpha_1 = \alpha_2 = \alpha_{1,3}$ angles. For each chosen point, the polarization response is also recorded for the input laser at frequency $\omega_1$, as described in Subsection 3.D, in order to characterize the local birefringence averaged over the whole thickness of the sample [29]. We repeat the same procedure for another $Z$ position of the sample [see Fig. 3(b)], by focusing the incident beams deeper into the collagen fiber, at a distance $Z = d$ from the bottom surface. In this case, both the incident and emitted beams are affected by birefringence. Polarization-resolved FWM measurements are then performed at the same in-plane ($X, Y$) position for different depths.

D. Experimental Polarization Distortions
The optical apparatus, in particular the dichroic filter recombining the excitation beams, may introduce some distortions in the incident polarizations. We use two photon fluorescence of a solution of Rhodamine 6G to characterize the ellipticity and dichroism of the incident polarizations, according to [36]. The parameters obtained (and used in all fitting procedures below) are 144° ellipticity and 1.09 dichroism factor between the $X$ and $Y$ field directions at $\lambda_1 = 724 \, nm$, and 55° ellipticity and 1.04 dichroism at $\lambda_3 = 857 \, nm$.

Moreover, collagen fibers are highly birefringent, which can lead to an erroneous interpretation of the collected signal and, by consequence, a wrong determination of the sample properties. This anisotropy is completely characterized by two parameters: (i) the orientation of the fast optical axis of the fiber $\Theta_b$, and (ii) the phase shift between its fast and slow optical axes $\Phi_0(d)$, after the field penetrates a distance $d$ into the sample. These birefringence parameters, defined in Fig. 3, can be estimated from a polarimetric method described in [29]. Briefly, we rotate the linear polarization angle $\alpha_1$ of the incident field $E(\omega_1)$ from 0° to 360° and record the transmitted intensity at the laser wavelength $\lambda_1$ along the $X$ and $Y$ directions, after the beam has traversed the whole thickness of the fiber $d = L$. The birefringence parameters, averaged over the whole sample thickness, can be determined from a fit of the obtained data, as described in Subsection 4.A. The birefringence of the sample at wavelength $\lambda_3$ is considered identical, because the sample does not exhibit resonances in this wavelength regime.

4. RESULTS
FWM images of a collagen fiber sample, acquired at $Z = 0 \, \mu m$, are shown in Fig. 4. The three images correspond to different regions of the fiber, distant by a few millimeters, which exhibit visibly different local fiber orientation. The three depicted points (labeled, respectively, positions 1, 2, and 3) represent the ($X, Y$) spot positions chosen to perform polarization-resolved measurements.

A. Determination of the Sample Birefringence
Figure 5(a) shows the intensities of the degenerate field at frequency $\omega_1$, transmitted throughout the fiber and detected along the $X$ and $Y$ axes, as a function of $\alpha_1$, for the position 3 of the sample. Considering that the collagen fiber acts as a single birefringent plate of thickness $L$, $\Theta_b$ and $\Phi_b(L)$ can be determined by a fitting of these polarization dependencies, using the method developed in [29]. This fit accounts for the instrumental distortions mentioned in Subsection 3.D. Figure 5(b) depicts the fitting cartography of the mean square error (MSE) as a function of $[\Theta_b, \Phi_b]$ for the data of Fig. 5(a). As already pointed out in [29], the solution $[\Theta_b, \Phi_b(L)]$ is not unique when polarization distortions are already present in the experimental setup: there are four distinct solutions.
of the molecular assemblies in the collagen fiber, as well as the mean orientation of the molecular distribution in the fiber, with respect to the macroscopic coordinate system. The fitting parameters are (i) the order parameters $f_2$ and $f_4$ (normalized by the isotropic contribution $f_0$) and (ii) the angle $\phi_0$ introduced in Eq. (4), which corresponds to the orientation of the molecular distribution in the $X-Y$ plane.

The fitting procedure consists of finding simultaneously, for the two polarization configurations described in Subsection 3.A ($\alpha_1$ varying with $\alpha_3 = 0^\circ$ and $\alpha_3$ varying), the set $(f_2, f_4, \phi_0)$ that minimizes the MSE function for the FWM intensities in the $X$ and $Y$ directions, normalized by the maximum of the total intensity, $I_X + I_Y$. With such definitions, the MSE function is given as follows:

\[
\text{MSE}(f_2, f_4, \phi_0) = \frac{1}{N_0} \sum_i \left[ \left[ T_X^\text{th}(\alpha_3, f_2, f_4, \phi_0) - T_X^\text{exp}(\alpha_i) \right]^2 + \left[ T_Y^\text{th}(\alpha_3, f_2, f_4, \phi_0) - T_Y^\text{exp}(\alpha_i) \right]^2 \right] + \left[ T_Y^\text{th}(\alpha_3, f_2, f_4, \phi_0) - T_Y^\text{exp}(\alpha_i) \right]^2,
\]

where the sum runs over the different incident polarization angles $\alpha_i$. The first two terms in the sum correspond to the first polarization configuration ($\alpha_1$ rotates and $\alpha_3 = 0^\circ$), while the last two terms stand for the second configuration ($\alpha_1 = \alpha_3 = 1^\circ$ rotate simultaneously). The superscript “th” stands for the theoretical intensity calculated from Eq. (5),

B. Orientation and Symmetry Order Properties of Collagen Fibers at $Z = 0 \mu m$

Fitting the polarization responses of the FWM signal (Section 2) allows characterization of the symmetry order of the molecular assemblies in the collagen fiber, as well as the mean orientation of the molecular distribution in the fiber, with respect to the macroscopic coordinate system. The fitting parameters are (i) the order parameters $f_2$ and $f_4$ (normalized by the isotropic contribution $f_0$) and (ii) the angle $\phi_0$ introduced in Eq. (4), which corresponds to the orientation of the molecular distribution in the $X-Y$ plane.

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\]

where the sum runs over the different incident polarization angles $\alpha_i$. The first two terms in the sum correspond to the first polarization configuration ($\alpha_1$ rotates and $\alpha_3 = 0^\circ$), while the last two terms stand for the second configuration ($\alpha_1 = \alpha_3 = 1^\circ$ rotate simultaneously). The superscript “th” stands for the theoretical intensity calculated from Eq. (5),

\[
\text{MSE}(f_2, f_4, \phi_0) = \frac{1}{N_0} \sum_i \left[ \left[ T_X^\text{th}(\alpha_3, f_2, f_4, \phi_0) - T_X^\text{exp}(\alpha_i) \right]^2 + \left[ T_Y^\text{th}(\alpha_3, f_2, f_4, \phi_0) - T_Y^\text{exp}(\alpha_i) \right]^2 \right] + \left[ T_Y^\text{th}(\alpha_3, f_2, f_4, \phi_0) - T_Y^\text{exp}(\alpha_i) \right]^2,
\]

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\]
with the FWM susceptibility obtained from Eqs. (3) and (4). The superscript “exp” corresponds to the FWM intensity acquired experimentally. Finally, the error function is normalized by the number of the incident polarization angles over which the sum is done, here $N = 73$ (this number is set to ensure a sufficient signal-to-noise ratio in the experimental data). This expression shows that the estimation of the measured parameters $(f_2, f_4, \phi_0)$ relies on constraining conditions that leave no ambiguity on their determination: indeed, it relies on the simultaneous fitting of two set of experiments, both measured along the $X$ and $Y$ directions.

Note that before fitting, the excitation and emitted field polarizations must be corrected for the polarization distortions introduced by the optical setup and by the birefringence of the sample, as described in Subsection 3.D. In the present situation, because the incident beams are focused at the bottom surface of the sample ($Z = 0 \mu m$), only the emitted field is affected by the sample birefringence. In this way, the theoretical FWM field used in Eq. (6) must take into account the orientation of the optical axis $\Theta_0$ and the birefringence phase shift $\Phi_0$ between the fast and slow axes of the collagen fiber. As we already pointed out in Subsection 4.A, when the incident fields are affected by dichroism and ellipticity, the solution $(\Theta_0, \Phi_0)$ is not unique. The two independent solutions do not result in the same theoretical FWM intensities, for the same parameters $(f_2, f_4, \phi_0)$. Here, we fit independently the parameters $(f_2, f_4, \phi_0)$ for both birefringence solutions that are independent and we choose the couple $(\Theta_0, \Phi_0)$ that gives the smallest MSE. Figure 6 shows the experimental results and best fits for the three positions depicted in Fig. 4. The retrieved parameters found using this procedure are summarized in Table 1, together with the birefringence angles $(\Theta_0, \Phi_0)$ used for these fits. For the three positions explored, the best solution for the birefringence parameters is solution 1 [$(\Theta_0, \Phi_0)_1$], defined in Subsection 4.A, which gives identical results as its equivalent solution 3 [$(\Theta_0, \Phi_0)_3$]. Note that not accounting for birefringence and instrumental polarization distortions induces a strong decrease in the fit quality, together with a bias on the obtained fitting parameters.

Once the parameters $f_2$ and $f_4$ are determined, it is possible to build the corresponding even-term truncation of the orientational distribution functions. Their 3D plots in the microscopic frame $(x, y, z)$ are shown in Fig. 7. In the three cases $f_4 \neq 0$, which means that the angular distribution has a high-order symmetry (hexadecapolar) contribution represented by a four-lobe shape. Position 2, which shows the less defined fibrillar structure on the FWM image (Fig. 4), exhibits the most isotropic molecular angular distribution with the lowest values for $f_2$ and $f_4$. Note that a complete view of the orientational distribution function $f(\theta)$, until the fourth order of the series expansion would require the knowledge of the order parameters $f_2$ and $f_4$, which could be probed by PSHG. The diversity of shapes found in this sample could be most likely due to the different arrangements of fibers depending on the sample location: indeed, we do not probe here single isolated crystalline collagen fibrils, but rather a macroscopic organization of them in fiber bundles that constitute the tendon tissue. Nevertheless, the shape found here for the even-order truncated distribution is compatible with the picture found for collagen in tissues, supposed to be

![Figure 6](image-url)  

**Fig. 6.** FWM intensities along $X$ (black) or $Y$ (gray) as a function of the incident polarization at $Z = 0 \mu m$. (a) $\alpha$ rotates and $\alpha_3 = 0^\circ$. (b) Both polarizations $\alpha_1$ and $\alpha_2$ rotate simultaneously. Solid lines, theoretical intensities given by the best fit; dots, experimental data. From left to right, the polar plots correspond, respectively, to positions 1, 2, and 3 (Fig. 4).

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<th>Position</th>
<th>$f_2$</th>
<th>$f_4$</th>
<th>$f_4/f_2$</th>
<th>$\phi_0$ (deg.)</th>
<th>$\Phi_0$ (deg.)</th>
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</table>

*Values of $(\Theta_0, \Phi_0)$ are obtained by fitting the sample's birefringence.

Table 1. Set of Parameters $(f_2, f_4, \phi_0)$ Obtained by the Fitting Procedure, for Different Positions of the Collagen Fiber in the Same $X$–$Y$ Plane for $Z = 0 \mu m$**
composed of nonlinear-active molecules lying along a cone surface pointing in the fiber direction [16,18].

The obtained results are represented schematically in Fig. 4, where the black lines superimposed to the FWM images of collagen represent the projection in the X–Y plane of the deduced molecular distribution oriented at \( \phi_0 \). The orientation of the local collagen fiber, observed from the acquired images, agrees qualitatively with the molecular averaged orientation \( \phi_0 \), for the three studied positions. The dashed lines in Fig. 4 correspond to the orientation of the sample fast optical axis \( \Theta_b \). This direction, which is deduced from an average information over the whole thickness of the collagen fiber, does not necessarily coincide with its local direction (black line). This is a signature of the heterogeneity of the sample along the Z direction. The measured birefringence phase shift measured in the three positions \( \Phi_b(L) = 180 - 200 \) (Table 1) agrees well with the expected thickness of the sample (\( L = 100–140 \) \( \mu m \)).

In the next section, we study the influence of the sample’s birefringence on the polarimetric FWM responses, when performing measurements at larger depths (\( Z > 0 \) \( \mu m \)) in the sample.

C. In-Depth Studies of Collagen Fibers: \( Z > 0 \) \( \mu m \)

In nonbirefringent samples, one would expect at a given position in the X–Y plane the same polarimetric FWM responses for different focal plane depths \( Z \). Figure 8 shows the experimental polarization-resolved FWM signals acquired at \( Z = 60 \) \( \mu m \), for the previous (X, Y) positions 1, 2, and 3. These polarimetric patterns are clearly different from the ones observed at \( Z = 0 \) \( \mu m \) (Fig. 4). A major change is verified at positions 1 and 3, especially for \( I_X \) recorded for a varying polarization \( \alpha_1 \). Inhomogeneities and different orientations of the fibrils along \( Z \), but also birefringence, could explain the changes observed. Indeed, at \( Z = 60 \) \( \mu m \), both the incident and emitted fields are affected by the effect of the fiber’s anisotropy, contrary to the case \( Z = 0 \) \( \mu m \) where only the emitted FWM undergoes birefringence modifications.

Figure 9 depicts theoretical polarization-resolved FWM responses, when the incident beams are focused at different depths \( d \) into the fiber. The incident and emitted fields are each affected by an effective birefringence whose phase shift is given, respectively, by \( \Phi_{inc} = d/L \Phi_b \) and \( \Phi_{em} = (1 - d/L) \Phi_b \). The theoretical FWM polar plots are built with the set of parameters \( f_2, f_3, \phi_0 \) obtained previously for position 3 and for both birefringence solutions 1 \( \{\Theta_b, \Phi_b\}_1 \) and 3 \( \{\Theta_b, \Phi_b\}_3 \), coupled by a \( \pi/2 \) periodicity for \( \Theta_b \). Contrary to the previous case \( Z = 0 \) \( \mu m \), for intermediate penetration depths (0 < \( Z = d < L \)) solutions 1 and 3 are seen to be no more equivalent in terms of polarization-resolved FWM responses; therefore, they have to be checked separately.

A direct qualitative comparison of these theoretical curves can be done with the experimental data of Fig. 8. In the case of the polarization scheme where only \( \alpha_1 \) rotates [Figs. 9(c), 9(d)], a four-lobe shape response appears for \( I_X \) [similar to what is observed experimentally in Fig. 8(a)] when \( d/L \leq 0.5 \) and \( \Theta_b = (\Theta_b)_3 = 148^\circ \). In the case where both polarizations \( \alpha_1 \) and \( \alpha_3 \) rotate simultaneously, a rough qualitative agreement with the experimental data [Fig. 8(b)] is found for \( d/L \leq 0.75 \) and \( \Theta_b = (\Theta_b)_3 \). We deduce therefore that solution 3 of the birefringence parameters is the most appropriate one and that the explored position is close to a depth between 0.5L and 0.75L, which is expected from the experimental setting \( Z = 60 \) \( \mu m \). Finally, the fact that the theoretical and experimental curves agree qualitatively well means that the molecular order parameters \( f_2, f_3, \phi_0 \) at this explored depth are likely to be close to the ones found previously.

Fig. 7. 3D plots, in the microscopic frame, of the even-order terms of the multipolar expansion of the molecular angular distribution functions, built from the fitted parameters \( f_2 \) and \( f_3 \) for positions 1, 2, and 3 (from left to right).

Fig. 8. Experimental FWM intensities, measured at \( Z = 60 \) \( \mu m \), along X (black) or Y (gray), as a function of the incident polarization. (a) \( \alpha_1 \) rotates and \( \alpha_3 = 0^\circ \). (b) Both polarizations \( \alpha_1 \) and \( \alpha_3 \) rotate simultaneously. From left to right, the polar plots correspond, respectively, to positions 1, 2, and 3.
for $Z = 0$. We find similar results for the other positions 1 and 2 of the sample.

Note however that the obtained theoretical plots agree only qualitatively with the experimental responses. Possible reasons for discrepancies can be (i) the fact that the local orientation $\phi_0$ of the collagen fiber is not homogeneous along $Z$ (in this case, $\Theta_0$ and $\Phi_0$ can no longer be considered as a constant parameters throughout the whole thickness of the collagen fiber) and (ii) the fact that the order parameters $(f_2, f_3)$ vary throughout the thickness of the sample. Determining the local symmetry and orientation of the molecular orientational distribution function is therefore a delicate procedure, especially in heterogeneous media. This is particularly true when the polarimetric measurements are carried out at intermediate depths of the sample, other than at its surfaces ($0 < Z = d < L$). Indeed, at $Z = 0 \mu m$, only the emitted signal is affected by birefringence, which can be measured separately throughout the whole thickness of the sample and introduced in the theoretical model, without affecting the determination of the order parameters and orientation of the molecular distribution (see Subsection 4.B). Determining molecular order orientation and symmetry information at intermediate focusing depths would in principle require to introduce a complete fit including all the parameters $(f_2, f_4, \phi_0, \Theta_0, \Phi_0)$; however, this large number of fitting parameters can lead to independent solutions difficult to discriminate. In the present work, we chose to introduce the local birefringence of the sample at different $Z$, $[\Theta_0(Z), \Phi_0(Z)]$, as a priori known parameters. A more accurate analysis could be performed by an iterative method consisting in performing the present analysis at increasing depths by small steps between $Z = 0$ and $Z = L$. At each depth step, the fit on the whole set of $(f_2, f_4, \phi_0, \Theta_0, \Phi_0)$ parameters could be performed using initial parameters obtained from the fit performed at the previous depth step, removing fitting solution ambiguities.

In addition to birefringence properties, a tissue can exhibit scattering and diattenuation, which should come into play in the physical interpretation of the nonlinear interaction with the collagen fibers. Following a procedure identical to [30], we noticed that the addition of such parameters does not affect or improve significantly the fits of the measured polarization-resolved FWM responses, which means that birefringence is the dominant process responsible for the distortion of these responses at increasing depths in the sample. In general, these parameters should be in principle added, at best from a preliminary characterization based on ellipsometry performed at the incident and FWM wavelengths.

In summary, we notice that in highly birefringent samples (possibly affected in addition by scattering or diattenuation), the choice of more than one configuration polarization together with a polarized detection is determined for the interpretation of the polarimetric data. For instance, in the presence of strong birefringence, the changes in the polar plots acquired at different depths in the sample, observed in Figs. 6 and 8, are more prominent for the intensities acquired along the $X$ direction and for the polarization scheme in which only $\alpha_1$ rotates. The presence of birefringence adds new unknowns to the problem of determining the molecular order of a medium and therefore requires a greater number of data. Nevertheless, this multiple configuration allows us, in principle, to explore cases where the local sample orientation contains out-of-plane components ($\theta_0$), because the number of parameters is still, in this case, compatible with the number of measurements performed. Finally, in nonbirefringent samples, the analysis can be considerably simplified. Indeed, in this situation only the detection of the total FWM intensity $(I_X + I_Y)$ in the case where both polarizations rotate simultaneously would be sufficient to determine the parameters $(f_2, f_4, \phi_0)$ without ambiguity.

5. CONCLUSION

We have shown that polarization-resolved FWM is a powerful technique to retrieve the even orders of symmetry up to the fourth order in a molecular statistical ensemble, such as for thick collagenous tissues. In particular, we propose a fitting procedure that allows estimating the microscopic molecular orientational distribution function and its orientation in the
macroscopic frame. Careful analysis concerning the birefringence of the sample must be performed before fitting the experimental FWM signals, in order to avoid erroneous or biased results. This microscopy technique, enabling imaging of local symmetry orders and molecular organization, brings new possibilities for high-contrast structural spatial investigation with submicrometric resolution. Polarization-resolved multimodal nonlinear microscopy can also be performed, by combining FWM with second-order nonlinear optical processes, such as SHG, in order to obtain complimentary information on the symmetry orders of the molecular distribution (in a nonresonant regime, one would expect the same microscopic structures to be resonsible for both SHG and FWM signals). Finally, this method can be enlarged to resonant polarization-resolved nonlinear techniques, such as CARS, by probing specific vibrational resonances of the molecular ensembles and allowing an imaging tool combining both structural and chemical selectivity.

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