Molecular orientation sensitive second harmonic microscopy by radially and azimuthally polarized light

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Abstract: We demonstrate the possibility to switch the z-polarization component of the illumination in the vicinity of the focus of high-NA objective lenses by applying radially and azimuthally polarized incident light. The influence of the field distribution on nonlinear effects was first investigated by the means of simulations. These were performed for high-NA objective lenses commonly used in nonlinear microscopy. Special attention is paid to the influence of the polarization of the incoming field. For linearly, circularly and radially polarized light a considerable polarization component in z-direction is generated by high NA focusing. Azimuthal polarization is an exceptional case: even for strong focusing no z-component arises. Furthermore, the influence of the input polarization on the intensity contributing to the nonlinear signal generation was computed. No distinct difference between comparable input polarization states was found for chosen thresholds of nonlinear signal generation. Differences in signal generation for radially and azimuthally polarized vortex beams were experimentally evaluated in native collagen tissue (porcine cornea). The findings are in good agreement with the theoretical predictions and display the possibility to probe the molecular orientation along the optical axis of samples with known nonlinear properties. The combination of simulations regarding the nonlinear response of materials and experiments with different sample orientations and present or non present z-polarization could help to increase the understanding of nonlinear signal formation in yet unstudied materials.

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References and links
1. R. M. Williams, W. R. Zipfel, and W. W. Webb, “Multiphoton microscopy in biological research,” Chem. Biol. 5, 603–608 (2001).
2. W. Denk and K. Svoboda, “Photon upmanship: Why multiphoton imaging is more than a gimmick,” Neuron 18, 351–357 (1997).
3. W. R. Zipfel, R. M. Williams, and W. W. Webb, “Nonlinear magic: multiphoton microscopy in the biosciences,” Nature Biotechnol. 21, 1369–1377 (2003).
4. F. Helmchen and W. Denk, “Deep tissue two-photon microscopy,” Nature Methods 2, 932–940 (2005).

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Multiphoton microscopy offers the possibility of high-resolution imaging at a cellular scale and has become a powerful tool in medical diagnosis during the last years [1–5].

For image formation signals with a nonlinear nature like two-photon induced fluorescence, second-harmonic generation (SHG) and third-harmonic generation (THG) are used [6, 7]. All these effects only occur when irradiated field strengths are of the order of the interatomic field strength. For this reason, objective lenses with a high numerical aperture (NA) are used in multiphoton microscopes to create a small focal volume in the range of less than 1 femtoliter leading to a very high field intensity [3]. Due to the large divergence of the focused beam,
the intensity outside of the focus quickly drops below the necessary value to induce nonlinear effects. Thus, the nonlinear effects are restricted to the focal volume and the signal generation is spatially localized [8].

In general, nonlinear effects are based on a nonlinear relationship between the exciting electric field and the polarization generated in the sample. The SHG process is based on the nonlinear polarization term of second order whereas THG and two-photon induced fluorescence are based on the third order term [9]. Thus, the nonlinear response of the medium is dependent on the second- and third-order nonlinear susceptibility \( \chi^{(2)} \) and \( \chi^{(3)} \) respectively. For many crystals and solid media \( \chi \)-tensors are widely studied and known [10]. While second harmonic generation is often used to image biological samples, there is still a lack of understanding of the signal formation. Especially the \( \chi \)-tensor of the specimen remains elusive [11–13].

The goal of this work is to develop tools to better understand the nonlinear signal generation in biological tissue. It is essential to know the field distribution in the focal region to understand the nonlinear response of the medium. The ability to probe the molecular orientation relative to a fixed axis in the focal plane by polarization resolved measurements has already been shown for materials with known \( \chi \)-tensor [12]. The possibility to probe the degree of molecular alignment parallel to the optical axis would add another piece to the puzzle. It is therefore necessary to be able to change the strength or entirely switch the z-polarization component of the illumination. Prior work shows that radial and azimuthal input polarization could fit this need: Important work concerning the influence of radial and azimuthal polarization in microscopy was done by Dorn et al. and first published in 2000 [14]. In 2003, Biss and Brown [15] investigated the effects of radial and azimuthal polarization in surface second harmonic generation at smooth metal and semiconductor surfaces and thin films. Basing on these first studies (among others), Yew and Sheppard [16] and Yoshiki et al. [17, 18] showed the application of radial polarization to study the orientation of collagen fibrils.

The presented work further investigates the effects of radial and azimuthal polarization in second harmonic generation at collagen fibrils. This was experimentally realized using polarized vortex beams. To fully understand the field distribution within the focal volume of a high-NA objective lens, we conducted simulations for typical laser beam parameters. Using the Vector Debye Theory, statements about the focal field distribution for different input polarizations, input field distributions and numerical apertures were made. The results of the simulations were tested and the ability to analyze the molecular orientation along the optical axis is shown for collagen in porcine cornea (native biological sample).

2. Vector Debye Theory

When using high-NA objective lenses for focusing light, effects like apodization and depolarization occur inside the focal volume of a laser beam. The Vector Debye Theory allows a description of the electric field vector \( \mathbf{E}_{\text{foc}} \) in a point \( P \) which is located in the vicinity of the focal point of the objective lens. The origin of coordinates \( O \) coincides with the position of the focal point. As an approximation of a microscope objective lens a single lens \( L \) behind an aperture \( A \) was used. For simplification the lens and the aperture are thought to be in the same \( z \)-plane (see Fig. 1).

An arbitrary incoming electric field \( \mathbf{E}_{\text{in}} \) is modified by the aperture and reaches the lens as \( \mathbf{E}_{\text{apt}} \). The lens forms a spherical wavefront with its center in the focal spot. The electric field is projected onto \( \mathbf{E}_{\text{proj}} \). In accordance with Leutenegger et al. [19] \( \mathbf{E}_{\text{proj}} \) can be calculated as
Fig. 1. Scheme of the optical arrangement: the objective lens is approximated by an aperture and a single lens. Aperture and lens are thought to be in the same z-plane (dotted points are not depicting an actual distance). The electrical field of the incoming light $E_{\text{in}}$ (plane wave approximation) is modified to $E_{\text{apt}}$ by the aperture. $E_{\text{apt}}$ is split up into its components in radial and azimuthal direction $E_p$ and $E_s$ respectively. The lens with focal distance $f$ focuses the light onto the origin of coordinates $O$. The field vector $E_p$ is deflected by $\theta$ and projected onto $E_r$. The electric field behind the lens $E_{\text{proj}}$ is the sum of $E_r$ and $E_s$. The new wavefront is a spherical cap. (Ref [19], Fig. 1)

$E_{\text{proj}} = M \cdot E_{\text{apt}}$ with Matrix $M$:

$$M = \begin{pmatrix}
\cos^2 \varphi \cos \theta + \sin^2 \varphi & \sin \varphi \cos \varphi \cos \theta - \sin \varphi \cos \varphi & 0 \\
\cos \varphi \sin \varphi \cos \theta - \sin \varphi \cos \varphi & \sin^2 \varphi \cos \theta + \cos^2 \varphi & 0 \\
\cos \varphi \sin \theta & \sin \varphi \sin \theta & 0
\end{pmatrix}$$

where the angles $\varphi$ and $\theta$ are defined as in Fig. 1. The field near the focal point $E_{\text{foc}}$ is a superposition of the fields $E_{\text{proj}}$ [20]:

$$E_{\text{foc}}(P) = \frac{ikf}{2\pi} \int_{0}^{2\pi} \int_{0}^{\alpha} E_{\text{proj}}(\varphi, \theta) \sqrt{\cos(\theta)} \exp[-ik(x + y + z)] \sin(\theta) \, d\theta \, d\varphi$$

where $\alpha$ is the maximum angle of convergence (NA = $n_{\text{med}} \sin \alpha$) and $s = \frac{k}{|k|}$ are the possible directions of the wave vector:

$$s = \begin{pmatrix}
\cos \varphi \sin \theta \\
\sin \varphi \sin \theta \\
\cos \theta
\end{pmatrix}$$

By substituting $\sin \theta \, d\theta \, d\varphi = \frac{1}{k^2 \cos \theta} \, dk_x \, dk_y$ and setting $E_{\text{proj}} \equiv 0$ for $f \sin \theta > R$ Eq. (2) can be written as a fourier transformation [19]:

$$E_{\text{foc}}(P) = \frac{if}{k} \mathcal{F} \left[ \frac{E_{\text{proj}}(\varphi, \theta)}{\sqrt{\cos(\theta)}} \exp[-ik_z z] \right]$$

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With both calculation methods it is possible to determine the field distribution within the focal
volume taking the polarization of the incoming field into account. Using the Fourier transform-
ation Eq. (4) reduces the computation time drastically.

An overview of the different polarization states used in the following sections is given in
Fig. 2. Circular polarization is not displayed separately as it can be described as rotating linear
polarization. At a given point in time and space, it is equal to linear polarization but rotates as
time progresses. Obviously, linear as well as circular polarization are spatially homogeneous
whereas radial and azimuthal polarization are spatially inhomogeneous polarization states.

Fig. 2. Schematic drawing of the electrical field distribution for linear, radial, radial vortex,
azimuthal and azimuthal vortex polarization. Except of the two vortex cases all field
distributions were assumed to be homogeneous.

3. Theoretical results

All calculations and simulations are based on the following parameters: 800 nm wavelength (\(\lambda\)),
80 mW power (cw and average power (\(P_{\text{avg}}\)) for pulsed case), 15.12 mm back focal plane of
the objective lens, a numerical aperture of 1.05 and a refractive index of 1.33 for the immersion
medium (water). These parameters coincide with our setup parameters.

3.1. Influence of the pulse length

To generate nonlinear effects pulsed lasers are advantageous because of their high pulse peak
powers. A key feature of a pulse is its spectral distribution which is directly connected to the
pulse duration via the time-bandwidth product \(\Delta \tau \Delta \nu \geq C\), where \(C\) is a constant depending on
the pulse shape [21]. For simulation pulses with a gaussian shape \((C = 0.4413)\) and with pulse
durations of 10 fs, 100 fs and 1000 fs were used. The spectral distribution of a laser pulse was
discretized at 32 points in a range \(\omega_0 \pm \Delta \Omega\), where \(\omega_0\) is the central frequency of the pulse
and \(\Delta \Omega\) stands for the full width at half maximum (FWHM) of the intensity distribution. For
simulation the pulsed case (a repetition rate of 80 MHz was assumed and is typical for tita-
nium:sapphire laser oscillators) was compared to a cw-laser. Thereby, the field distribution was

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set to be gaussian and the polarization was chosen to be linear. Due to the different focusing characteristics of the different spectral components, the field distribution of the pulses in the focus differs from the monochromatic cw-case. Summing up the spectral intensity components at each point in space and subsequent time averaging leads to $I_{\text{avg}}(x,y)$. The latter quantity was then compared pointwise to the cw-intensity $I_{\text{cw}}(x,y)$. In the end, the intensity distribution along the lateral axis (perpendicular to the polarization axis) inside the focal plane was compared for the two cases. The results are shown in Fig. 3 where $\Delta I$ stands for the relative deviation between the cw and pulsed case and is given in %. Specifically, $\Delta I$ is calculated by Eq. (5):

$$\Delta I(x,y) = \frac{I_{\text{cw}}(x,y) - I_{\text{avg}}(x,y)}{I_{\text{peak}}(0,0)}$$ (5)

The difference between the cw- and pulsed case is less than 1 %. This helps to simplify any further calculations and simulations: the spectral distribution has not to be taken into account and it is sufficient to consider cw-mode in order to calculate different polarization components in the focus.

![Fig. 3. Comparison between cw and pulsed case: The relative deviation between the cw and pulsed case is less than 1 %. Thus, calculations can be based on the cw-case.](image)

### 3.2. Polarization of the incoming field

Most of the laser sources used in the field of multiphoton microscopy have a linear output polarization which is commonly not changed and used for signal generation. Accordingly the simulation was first carried out for a linear polarization at the back aperture of the objective lens. In a next step, the polarization distribution inside the focal volume was simulated for light of various incoming polarization states. Except for the vortex case, the input field distribution was assumed to be homogeneous. For all presented results the distribution of different polarization directions was calculated in the following way:

$$x - \text{direction} = \frac{\sum I_x}{\sum I}, \quad y - \text{direction} = \frac{\sum I_y}{\sum I}, \quad z - \text{direction} = \frac{\sum I_z}{\sum I}$$ (6)

The calculated results based on Eq. (6) are shown in Table 1 where $I_{\text{max}}$ is the local maximum value of the time averaged intensity.

For linearly polarized light a change of the polarization direction can be observed. Most of the light (82.6%) stays polarized like before after focusing, only 0.6% are converted into the perpendicular polarization direction, but 16.8% are converted into the z-direction. Because of radial symmetry the results for left and right circularly polarized light are equivalent. Here, 16.8% are converted into the z-direction as well. For radial polarization the different components of
Table 1. Behavior of different incoming field polarizations after strong focusing (NA = 1.05, \(\lambda = 800 \text{ nm}\)). The field distribution was assumed to be homogeneous except for the two vortex beams. Every polarization state splits up into x-, y- and z-polarization components. For azimuthal polarization no z-component arises.

| Polarization       | \(I_{\text{max}}\) [W/cm\(^2\)] | FWHM x [nm] | FWHM y [nm] | x-Pol [%] | y-Pol [%] | z-Pol [%] |
|--------------------|----------------------------------|-------------|-------------|-----------|-----------|-----------|
| linear in x-direction | \(4.4 \times 10^7\) | 378         | 460         | 82.6      | 0.6       | 16.8      |
| linear in y-direction | \(4.4 \times 10^7\) | 460         | 378         | 0.6       | 82.6      | 16.8      |
| circular radial     | \(4.4 \times 10^7\) | 414         | 414         | 41.6      | 41.6      | 16.8      |
| azimuthal radial    | \(1.5 \times 10^7\) | 772         | 772         | 33.2      | 33.2      | 33.5      |
| vortex radial \((l=1)\) | \(2.4 \times 10^7\) | 914         | 914         | 50.0      | 50.0      | 0.0       |
| vortex azimuthal \((l=1)\) | \(1.7 \times 10^7\) | 396         | 396         | 37.0      | 37.0      | 25.9      |

Polarization split up into an almost homogeneous distribution for x-, y- and z-direction (around 33% for a homogeneous and 26% for a vortex beam). In contrast to radially polarized light, the behavior for azimuthally polarized light is completely different. Azimuthal polarization splits up into 50% in x-direction and 50% into y-direction. The components of in z-direction polarized light is 0% for this case. With respect to our experiments explained in section 4 two cases with a non-homogeneous input field were simulated: radially and azimuthally polarized vortex beams with a charge equal to \(l=1\). For these two cases the main result stays like mentioned before and the component of in z-direction polarized light is 0% when using azimuthal polarization (see Fig. 4).

![Intensity Log[I/I_{\text{max}}]](vortex_radial)

![Spatial distribution of polarization components after focussing radially and azimuthally polarized vortex beams with a high-NA objective lens (NA = 1.05, \(\lambda = 800 \text{ nm}\)). A z-component of azimuthally polarized vortex beams does not exist.](vortex_azimuthal)
Thus, imaging with both radially polarized incident light and azimuthally polarized incident light could allow to determine the degree of molecular orientation along the optical axis. The strong z-component arising for radially polarized light would lead to a change in signal strength compared to the response to azimuthally polarized light depending on the $\chi$-tensor.

In addition to the influence of the polarization distribution inside the focal volume, the incoming polarization also effects the size and shape of the focus. Figure 5 shows the results of the simulation with respect to the focal shape. The field distribution is zero in the center of the focal plane for a homogeneous azimuthally polarized incoming field. As a result, the FWHM for this polarization is more than twice as large as for linearly and circularly polarized light. In contrast, an azimuthally polarized vortex beam has its maximum in the center of the focal plane. Comparing the two vortex beam cases, azimuthally polarized light has a much smaller FWHM than radially polarized light.

![Fig. 5. Size and shape of the focus for different input polarizations (NA = 1.05, $\lambda = 800$ nm). The field distribution of the incoming light was assumed to be homogeneous except for the vortex cases. Except for the case of an azimuthally polarized homogeneous beam, all resulting intensity distributions have their maximum in the center of the focal plane.](image)

### 3.3. Thresholds for nonlinear signal generation

Radially polarized incident light can lead to a change in signal strength (compared to the response to azimuthally polarized light) due to the molecular orientation along the optical axis. But also the different intensity distributions might lead to disparities in the generated nonlinear signal and could diminish the informative value regarding the molecular orientation. Consequently, the influence of the focal shape on the nonlinear signal generation has to be evaluated to determine the effects solely due to the z-polarization component. For the simulation a stepwise threshold model was used. The simplification with respect to the threshold (stepwise not continuous) allowed fast computation of a quantity directly proportional to the generated signal. The calculations neglect all coherence effects. Especially phase matching due to the non collinear incident of the focussed light was not considered. Consequently, the results give a rough estimation of the influence of the focal field distribution on signal generation.

Nonlinear polarization is assumed to only occur when $|E(x,y,z)| \geq n_{NL}E_{at}$, where $E_{at} \approx 10^{11}$ V/m stands for the interatomic field strength and $n_{NL}$ is a scaling factor. This is equivalent to intensities of the order of $10^{15}$ W/cm$^2$. The threshold represents a level that has to be exceeded by the momentary intensity. Thus, it is not sufficient to only consider the
cw-case but rather the pulsed case. Laser pulse intensities were calculated based on the values for the cw-case presented in section 3.2. The intensity of the pulses was approximated by
\[ I_{\text{peak}} = I_{\text{avg}} / (R \tau_{\text{pulse}}) \]
where \( R \) is the repetition rate of the laser (80 MHz) and \( \tau_{\text{pulse}} \) is the temporal pulse width (140 fs). Second harmonic generation depends on the squared intensity of the fundamental beam \[10\]. Thus, to evaluate the influence of the focal field distribution, we calculated the sum over all contributing squared intensities for linearly, circularly, radially and azimuthally polarized light (homogeneous field distribution) and for radial as well as for azimuthal vortex beams. All intensities below the threshold value (different values used: \( n_{NL} \approx 10^{-3}-10^{-2} \)) were set to zero. Thereby, the result is directly proportional to the SH signal. Table 2 shows the results. For all chosen thresholds, the results for radial and azimuthal vortex beams are of the same order of magnitude.

| Polarization     | \( I_{\text{peak}} \) [W/cm²] | \( \sum I^2 \) \( 10^{27} \text{W}^2/\text{cm}^4 \) |
|------------------|---------------------------------|-----------------------------------------------|
|                  | \( I_{\text{th}} \) [W/cm²] : 0  | 5 \times 10^{10} | 5 \times 10^{11} | 1 \times 10^{12} |
| linear           | 3.90 \times 10^{12}             | 10.47  | 10.46  | 10.31  | 9.88   |
| circular         | 3.90 \times 10^{12}             | 10.34  | 10.33  | 10.17  | 9.73   |
| radial           | 1.37 \times 10^{12}             | 4.23   | 4.22   | 3.83   | 2.18   |
| azimuthal        | 1.25 \times 10^{12}             | 4.84   | 4.83   | 4.58   | 3.19   |
| vort. radial     | 1.51 \times 10^{12}             | 3.61   | 3.61   | 2.93   | 1.76   |
| vort. azimuthal  | 2.14 \times 10^{12}             | 4.07   | 4.07   | 3.15   | 2.56   |

### 3.4. Influence of the incoming beam shape

The beam shape is another critical parameter for the field distribution inside the focal volume. To investigate the influence of the beam shape simulations were carried out for six different cases: a homogeneous field, a field which is linearly dropping with increasing radius, a gaussian field (1/e-level at the aperture edges (R)), an overfilled back aperture with a gaussian beam (1/e-level at 1.75R), an annular shaped beam and a vortex beam. In each case the incoming field is linearly polarized. All other parameters remain unchanged. The input power is 80 mW for all cases but for the gaussian, overfilled gaussian, annular beam and the vortex beam the aperture cuts off the beam partially. Thus, the absolute intensity values do not allow any conclusions, as the transmitted power was not the same for the different cases. The results are shown in Fig. 6. As described before, the linear polarization is splitting up into components after focussing. All beam shapes lead to z-polarization components of 10-17 % after focussing, except the annular beam: for this case a strong z-polarization component of 29.5 % arises. This is due to the strong apodization of the outer components.

Due to the different focal shapes of the various beams differences in resolution will occur. This is beyond the scope of this work and thus not further investigated.
Fig. 6. Variation of the field distribution before focussing with an objective lens (NA = 1.05, \( \lambda = 800 \text{ nm} \)): the z-polarization component depends strongly on the incoming field distribution. All calculations were done for linear input polarization.

3.5. Influence of numerical aperture (NA)

The numerical aperture (NA = \( n_{\text{med}} \sin \alpha \)) of the objective lens crucially influences the size of the focus as well as the polarization distribution inside the focal volume. This influence was simulated for different numerical apertures with respect to the z-polarization component. The results are shown in Fig. 7.

With increasing NA the effect of depolarisation inside the focal volume is increasing. As shown before, an azimuthally polarized input field shows no z-polarization component after focussing - independent from the NA. Concludingly, high NAs lead to a small focal volume which results in a high resolution of the multiphoton microscope but the pure polarization state is getting lost with increasing NA.
4. Experimental results

In order to evaluate the theoretical results, experiments on native corneal tissue were carried out. The cornea is the foremost layer of the eye and builds the interface with the air. Like shown in many publications it is possible to create a strong second harmonic signal from the stromal region of cornea [22, 23]. This region mostly consists of fibrillar collagen which has a non-centrosymmetric molecular structure (helix) leading to strong SH-signals [24, 25]. Maximum SHG is reached for E-field vectors parallel to the collagen fibril [3]. Collagen fibrils are arranged interwoven like a net inside this region. In order to investigate the influence of the incoming polarization, z-stacks were recorded with radially polarized and azimuthally polarized vortex beams. This was done for two different orientations of the corneal tissue to determine if the z-polarization component is able to find the molecular orientation. In the following explanations light is characterized and named after the polarization state at the back aperture of the objective lens. The optical axis is the z-axis of the coordinate system.

4.1. Experimental setup and generation of radial and azimuthal input polarization

The fs-laser system Chameleon Ultra II (Coherent, Santa Clara, USA) was used in order to provide the laser light for the laser-scanning multiphoton microscope MPM200 (Thorlabs, New Jersey, USA). For focusing the beam into the sample a 25x objective lens (XLPL25XWMP, Olympus GmbH, Hamburg) was used. This objective lens is optimized for multiphoton applications in the near infrared. For all experiments a wavelength of 800 nm and an average power of approximately 80 mW in the sample plane were used. A scheme of the experimental setup is displayed in Fig. 8.

![Fig. 8. Experimental setup: The fs-laser power was controlled by a light valve (polarizing beam splitter cube (PBS) and a half-wave plate (λ/2). The beam diameter was then adjusted to the scanning mirrors using a telescope. The polarizing elements were placed right in front of the scanning unit. Two quarter-wave plates (λ/4) and a half-wave plate ensured circular polarization. The custom made waveplate converted left circular to a radially and right circular to an azimuthally polarized vortex beam. The polarization state could be checked optional with a linear polarizer in front of a CCD-camera. Behind the scanning mirrors another telescope widened the beam to slightly overfill the back aperture of the objective lens. The backward signal was deflected by the primary dichroic mirror and the signal was detected with a PMT after a secondary dichroic mirror and a band-pass filter.](image-url)
The laser system Chameleon Ultra II is emitting linearly polarized light. With two quarter-wave plates ($\lambda/4$) and one half-wave plate ($\lambda/2$) (arrangement: $\lambda/4-\lambda/2-\lambda/4$) this light was converted into circularly polarized light. In order to guarantee an almost ideal polarization state a polarimeter (PAX5720IR1-T, Thorlabs) was used behind these waveplates. By measuring the Stokes parameters the corresponding circular polarization state could be ensured.

Radially and azimuthally polarized light was created by a custom made phase plate. By passing through the phase plate, right circularly polarized light is converted to an azimuthally polarized vortex beam and left circularly polarized light to a radially polarized vortex beam respectively [26, 27]. The two exceptional polarization states could not be measured with the polarimeter. In order to investigate these polarization states a linear polarizer (transmission for y-directed E-field components) was used and an image of the beam was taken with a CCD camera. As displayed in Fig. 9, a horizontal dark line shows up in the image for the radial case and a vertical dark line for the azimuthal case respectively. This corresponds to the expected behavior for radially and azimuthally polarized vortex beams [26].

![Radial polarization](image1)

(a) Horizontal components are taken out by the polarizer. This leads to a horizontal dark line for radial polarized light on the CCD image.

![Azimuthal polarization](image2)

(b) Horizontal components are taken out by the polarizer. This leads to a vertical dark line for azimuthal polarized light on the CCD image.

Fig. 9. Comparison between radially and azimuthally polarized light. The present polarization states can be identified with a CCD camera and a linear polarizer in front of the camera.

Furthermore, the transmission efficiency of the entire microscope system was measured to be identical for both polarization states. Thus, the same power was deposited at the sample plane for radially and azimuthally polarized light in all experiments.

4.2. Cornea preparation

Eyes from fresh slaughtered pigs were obtained from the local slaughterhouse. The corneas were extracted and cut into small slices right before the experiment was conducted. Beforehand a special holder had been custom made (see Fig. 10): a flat metal ring was glued onto a microscope slide. For the experiment, the ring was filled with Vidisic gel (Bausch & Lomb GmbH, Germany) and a small slice of cornea was placed into the gel. Vidisic is an eye gel with a refractive index of nearly 1.33 and a high viscosity leading to enough stiffness to ensure the positioning of the cornea. Then a cover slip was placed on top of the ring and gel, closing the reservoir. On top of the coverslip, water was used as immersion medium. Water matches the index of the gel while the lower viscosity ensures that no movements of the objective lens are translated to the cornea. Thus, multiple z-stacks could be recorded without changing the position of the cornea leading to comparable data.
4.3. **SHG at collagen fibrils with radially and azimuthally polarized light**

Z-stacks were recorded for two different orientations of the extracted cornea. On the one hand for an upright position, equal to imaging the cornea from the sides, leading to a transverse view. In this case, the collagen planes (lamellae) are (approximately) parallel to the optical axis. On the other hand, the cornea was imaged from the front, leading to a coronal view. The collagen planes are (approximately) perpendicular to the optical axis. A scheme of the situation and exemplary images for each case are depicted in Fig. 11.

![Scheme of the two imaging modes.](image)

**Fig. 11.** Scheme of the two imaging modes. The orientation of the collagen planes relative to the optical axis is sketched and exemplary resulting pictures are shown: a) The optical axis is parallel to the collagen planes leading to a transverse view. b) The optical axis is perpendicular to the collagen planes leading to a coronal view. Scale bars: 30 µm.
The lamellae are made up of fibrils lying in the same plane without predominant orientation [12, 28]. For the transverse view this means a significant projection of the collagen molecular axis onto the optical axis. Contrary, there is only a negligible projection of the collagen molecular axis onto the optical axis in the case of the coronal view. The SH-signal was detected in backward direction. For each cornea orientation, two corresponding stacks at five different positions were recorded. The first stack was performed with azimuthally, the second with radially polarized incident light. All stacks were acquired starting near the surface and stopping at a depth of 100 µm (2 µm step size). Each image in the stack was averaged over ten frames. The pixel resolution was 1024 x 1024 and the field size 300 µm x 300 µm. Afterwards, the mean grey value was measured with ImageJ and the electronic noise of the detector was subtracted for every single image. Subsequently, the quotient of the mean grey values of the two images recorded at the same z-position (and the same lateral position) was computed. Furthermore, the mean value of the first five grey value ratios (equivalent to a depth of 8 µm) was computed for every position. The results are depicted in the graphs of Fig. 12. In the first case, for collagen planes approximately parallel to the optical axis, the mean grey values of the images taken with incoming radially polarized light are distinctly higher than for azimuthally polarized light. The mean ratio is: 1.23 ± 0.03. Even for a depth of 100 µm inside of the tissue, the ratio was significantly above 1. The average ratio (here of the mean of the last five z-positions) is 1.24 ± 0.03. In the second case, for collagen planes approximately perpendicular to the optical axis, the mean grey values of the images taken with incoming radially polarized light are nearly the same as for azimuthally polarized light over the whole course of the z-stack (0.99).

A normalization measurement to quantify the influence of the focal shape was performed with a homogeneous fluorescent sample (fluorescein in solution) under the same experimental conditions. No significant difference in signal strength for azimuthally polarized and radially polarized input light could be found (mean ratio of 25 measurements: 0.99 ± 0.02).

![Graph](image1.png)

(a) Transverse view: radially polarized incident light leads to a distinctly higher signal strength compared to azimuthally polarized light. The average over all 5 positions is 1.23 ± 0.03.

![Graph](image2.png)

(b) Coronal view: radially polarized incident light leads to approximately the same signal strength as azimuthally polarized light. The average over all 5 positions is 0.99.

Fig. 12. Ratios of the mean grey values of the images recorded with radially and azimuthally polarized vortex beams for (a) transverse and (b) coronal view. In each case the mean grey value of an image recorded with radially polarized light was compared to the mean grey value of the corresponding picture recorded with azimuthally polarized incident light. The ratios of the first 5 z-positions (equal to 8 µm in depth) were averaged for each position in the cornea.
5. Discussion

Using the Vector Debye Theory allowed us to calculate the field distribution behind high-NA objective lenses used in multiphoton microscopes. The incoming field polarization, beam shape and the NA of the objective lens were taken into account and their influence on the focal field distribution was studied.

The x-, y- and z-polarization components after focussing with a high-NA objective lens were calculated. Especially for non common polarization states in multiphoton microscopy like radial and azimuthal polarization a difference was found. While radially polarized light exhibits the strongest z-component (33.5% (homogeneous), 25.9% (vortex beam)) after focussing, azimuthally polarized light was determined to have no z-component. This is an essential result and gives the opportunity to switch the z-component on and off by choosing different polarization states for the incoming field. The simple threshold model allows to estimate the relative strength of signal generation for different polarization states. The sum over the squared intensities for linearly and circularly polarized light is almost the same for all thresholds. For azimuthally polarized light (homogeneous as well as vortex beam) the summed up squared intensities are for all thresholds higher than for radially polarized light but have the same order of magnitude.

The field distribution was further investigated for different beam shapes and the results show that the z-polarization component is strongly dependent on the incoming beam shape.

Data for the NA show that the higher the NA, the stronger the z-polarization component arises. Exceptional cases are present when working with azimuthally polarized light. The z-component vanishes completely and is thus independent from the NA. Nevertheless, high-NA objective lenses are necessary for multiphoton microscopy. They create a very small focal volume, but the pure polarization state is going to be lost.

The experiments on native porcine corneal tissue back the results of the simulation for different input polarizations. For flat cornea positioning (coronal view) which matches the in-vivo situation, (almost) all fibrils are parallel to the surface of the eyeball and thus in the x-y-plane. As stated in numerous publications, the fibrils are well aligned regarding a small section of the cornea but overall randomly distributed [12, 28]. Rotating the sample 90 degrees to an upright position (transverse view) changes the orientation of the lamellae/fibrils. Each lamella can now be regarded as a plane spanned by a z-vector and a vector of the x-y-plane describing the course of the lamella (see image in Fig. 11(a)). Consequently, almost all fibrils will be at least partially oriented along the z-axis. While the projection of the collagen molecular axis on the optical axis is negligible for the flat/coronal case, there is a distinct projection present in the case of the transverse view. Radially polarized light leads to distinctly higher backward SH-signals compared to azimuthal light (average ratio radial/azimuthal: 1.23 ± 0.03) for upright positioning (transverse view). For flat positioning (coronal view), no distinct difference in signal generation could be found (average ratio radial/azimuthal: 0.99).

The numerical values coming out of the experiment should not be overrated. Several factors play a role in the process of signal formation and transmission (scattering and absorption effects) of the light. The most crucial factor is the biological nature of the sample. It is unlikely that the collagen planes were all perfectly aligned either parallel or perpendicular to the optical axis. Hence, the results of course vary from position to position and over the course of the z-stack. Above all the pureness of the polarization state can not be taken as a given. The test with a linear polarization filter and a CCD camera showed that the custom made polarization plate did change the polarization to mainly radial or mainly azimuthal. Nevertheless it remains unknown how pure the state of polarization actually is. Another uncertainty factor is the development of the polarization state. Before the light actually hits the sample, it is deflected by several mirrors, including one dichroic. This could lead to phase distortions and thus changes in the state of polarization. However, phase distortions due to the dichroic mirror degrading the wavefront
as well as the polarization state were minimized by reducing mechanical tension on the mirror. The polarization of the probing light probably degrades for deeper imaging planes due to scattering and absorption processes in the tissue. In fact, the ratio varied with depth and showed a slight increase for averaging over the last five data points and the five positions (1.24 ± 0.03).

This is most likely due to a change in molecular orientation. An influence of the focal shape can be ruled out: A normalization measurement on a fluorescent sample showed no difference in signal strength for azimuthal and radial input light (mean ratio: 0.99 ± 0.02). Assuming a negligible influence of the polarization state on the fluorescence generation, the influence of the focal shape on the signal strength can be regarded as negligible. This is in good accordance (in the limits of the model accuracy) with the results of the simulation for low thresholds. Following, the distinct differences in SH signal strength for radially and azimuthally polarized light can be ascribed to the polarization change in the focal region. Consequently, switching the polarization between azimuthal and radial allows to determine the collagen molecular orientation along the optical axis. However, due to the described uncertainties only qualitative conclusions can be drawn and the degree of orientation remains unknown.

6. Conclusion

The presented theoretical and experimental results allow to conclude the following: the field distribution behind high-NA objective lenses can be computed by the means of Vector Debye Theory regarding the influence of several decisive parameters. The effect of apodization and the resulting z-polarization component in the focus are of special interest for second harmonic microscopy. The ability to switch the z-component using radially or azimuthally polarized light adds the third dimension to polarization sensitive second harmonic microscopy. The theoretical findings are in good agreement with the experimental results and show that collagenous samples can be investigated regarding the 3D fibril orientation. Further experiments with a well ordered collagen sample (e.g. rat tail tendon) could be done to allow quantifications of the degree of alignment with the optical axis. The presented technique can be extended to other collagenous tissues and might thus be useful in a variety of biomedical applications. Furthermore it could be used on other non centrosymmetric samples enabling the determination of yet unknown χ-tensors.

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