Measurement of orientation and susceptibility ratios using a polarization-resolved second-harmonic generation holographic microscope

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Abstract: Three-dimensional second-harmonic fields, sample orientation, and susceptibility ratios of biological samples are measured using polarization-resolved second-harmonic generation (SHG) microscopy. The three-dimensional (3D) polarization is gathered by measurement of a series of holograms for which excitation and analyzer polarizations are systematically varied, and the 3D SHG field is recovered through numerical back propagation. Harmonophore orientation is resolved in 3D from a sub-set of polarization-resolved SHG holograms. We further expand on previous approaches for the determination of susceptibility ratios, adding the calculation of multiple ratio values to allow intrinsic verification.

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1. Introduction

Second harmonic generation (SHG) provides rich information regarding structural proteins and their organization [1–3]. This structural specificity derives from the fact that SHG signal vanishes when inversion symmetry is present. This non-centrosymmetric organizational requirement gives rise to an intrinsic polarization dependence in SHG [4]. This polarization dependence allows for identification of the axes of symmetry and relative strengths of SH nonlinear.

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optical tensor components of harmonophores (SH generating structures), averaged over the focal volume of signal generation [5, 6].

In the context of laser-scanning second harmonic generation microscopy, this polarization sensitivity is exploited to obtain detailed structural information about biological tissues [7]. Based on models of the fibril organization, relationships between SHG intensity and incident linear polarization angle, orientation, and susceptibility components can be extracted through fitting a set of fundamental excitation angles. These analyses have been used to observe the orientation of collagen fibers [6], cellulose fibers [8], muscle fibers [9, 10], and cartilage [11]. Polarization SHG analysis can also discriminate between endogenous SHG sources from biological tissues [12] as well as probing molecular motor dynamics [13, 14]. In combination with detailed models, estimates of atomic level structure, such as the helix pitch angle of the dominant harmonophore bonds can be deduced [15]. Fits to the excitation polarization dependent SHG intensity are time-consuming because a large number of polarization excitation angles must be used. With clever choice of excitation angles, a significant reduction of the amount of data that must be captured is achieved by using phase-cycling algebra to efficiently compute the fiber orientation, susceptibility terms, and harmonophore bond pitch angles [16, 17].

Healthy functioning of many tissues relies on proper organization, which suggests the use of quantitative harmonophore orientation in tissues as a pathology indicator of certain diseases. This approach has been pursued for identifying healthy and pathological skin dermis [18]. It also allows for quantitative analysis of injured tendons [19]. Polarized SHG imaging is able to probe collagen fiber orientation in arterial walls, which may prove useful as a diagnostic of coronary disease [20]. The wide range of applications of polarized-SHG (PSHG) microscopy for use as a quantitative diagnostic tool for diseases and other pathological conditions can be found in Campagnola and Dong’s recent review [21].

PSHG is extremely powerful, and allows for observation of the three dimensional organization of structural tissues as the laser-scanning SHG microscopy employed restricts SHG signal generation to a small volume near the laser focus, providing nonlinear optical sectioning. 3D images are obtained by sequentially scanning a tight laser focus through a volume. The serial acquisition of each voxel used to construct a 3D image is a slow process, preventing application of SHG microscopy to rapid dynamics, restricting update rates in a 3D volume to well below 1 Hz. This presents challenges for the direct observation of 3D temporal behavior resolved through PSHG, such at those present for cardiac dynamics through measurement of intrinsic SHG signals, which could be used to study embryonic heart dynamics [22]. To address this deficiency, SHG holography has been recently developed [23–26]. Holography permits capture of 3D object information in a 2D image, drastically improving imaging speeds. As holography is an interferometric technique, only co-aligned polarizations will interfere. To date, SHG holography methods have selected one particular pair of excitation and recording polarizations.

In this paper, we develop a polarization-resolved SHG holographic microscope. With a set of SHG holograms, each with a specific excitation and recording polarization, we can reconstruct the orientation and susceptibility ratios of label-free, endogenous SHG generating tissue structures in 3D. In addition, we obtain polarization-resolved second harmonic phase images throughout the 3D image volume. To minimize the data that must be processed, we have adapted a phase-cycling approach analogous to that method pioneered by Odin et al. [16, 17] for used with PSHG. The resultant framework allows for computation of the total SHG intensity, and orientation of the local harmonophore. Our modified algorithm also admits several methods for computing the susceptibility ratios of the tensor elements from the SH-active tissue structures – providing a self-consistency check for the analysis.
2. **Theory**

SHG holography is an interferometric technique that records the amplitude and phase of a SHG object field and a more powerful reference field generated by a second harmonic crystal. The second harmonic object field is generated through second harmonic scattering by a weakly focused fundamental laser beam from SH-active harmonophores in a specimen. For a given incident fundamental field polarization, a second harmonic field is scattered with a polarization determined through tensor algebra that takes into account the incident fundamental field polarization, and the organization and orientation of the SHG harmonophore emitters in the specimen. Second harmonic hologram image formation is only sensitive to interference terms that occur through co-polarized components of the reference and object beams. Polarization-selection of interference presents an opportunity to fully resolve the polarization state of the SHG object field in three dimensions through recording and reconstruction of the amplitude and phase of a set of polarized-SHG holograms of a small set of 2D hologram images.

Once the polarization state of the object field is determined, standard numerical propagation routines provide a 3D reconstruction of the amplitude and phase of the SHG generation throughout the specimen volume. Further specimen information from polarized SHG data is possible by adopting a model of the local second-order nonlinear susceptibility tensor. As SHG requires media that lack inversion symmetry on both microscopic and macroscopic scales, SHG signals are restricted to tissues that display this organization. Prior work has studied the organization of many second harmonic generation active tissues. A number of groups have shown that local susceptibility tensor can be described in terms of hexagonal or cylindrical symmetry, as demonstrated for muscle [9, 15], collagen [27, 28], and starch [29].

Using the contracted notation $d_{ik} = 1/2\varepsilon_{0}\chi_{ik}^{(2)}$, the possible non-zero tensor elements are given as:

- $d_{33} = 1/2\varepsilon_{0}\chi_{xxx}^{(2)}$, $d_{31} = 1/2\varepsilon_{0}\chi_{xxz}^{(2)} = 1/2\varepsilon_{0}\chi_{zxy}^{(2)}$, $d_{15} = 1/2\varepsilon_{0}\chi_{xzz}^{(2)} = 1/2\varepsilon_{0}\chi_{xyz}^{(2)} = 1/2\varepsilon_{0}\chi_{zyy}^{(2)}$, $d_{14} = 1/2\varepsilon_{0}\chi_{xyz}^{(2)} = 1/2\varepsilon_{0}\chi_{yzy}^{(2)} = -1/2\varepsilon_{0}\chi_{yzx}^{(2)} = -1/2\varepsilon_{0}\chi_{zyx}^{(2)}$. Assuming Kleinman symmetry provides further simplification, with $d_{14} = 0$ and $d_{15} = d_{31}$. The ratio of the two remaining tensor components, $\rho = d_{33}^{15}/d_{31}$, gives information about the ordering and supramolecular organization.

While we are making use of femtosecond laser pulses of a few hundred femtoseconds in duration, we may theoretically describe the SHG hologram formation by considering a constant-wave (cw) fundamental field for illumination, $E_{in}$. This incident field is assumed to be linearly polarized along the $x$-axis of the transverse coordinate frame of the specimen. In a coordinate frame $(x, y, z)$ in the region of the specimen, where $z$ is the direction of propagation of the fields and assuming that the field is not localized strongly enough to give rise to a strong longitudinal field, we can write the SHG polarization density generated by an incident field polarized linearly along an angle $\alpha$ with respect to the $x$-axis by a fiber oriented at an angle $\theta$ again with respect to the $x$-axis as:

\[
P_{x}^{(2)}(r) = E_{in}^{2}\sin[2\alpha + 2\theta(r)]d_{31}c(r) \tag{1}
\]

\[
P_{y}^{(2)}(r) = E_{in}^{2}\left\{ \sin[\alpha + \theta(r)]^{2}d_{31} + \cos[\alpha + \theta(r)]^{2}d_{33} \right\}c(r) \tag{2}
\]

where $c(r)$ is the spatial distribution of the harmonophore concentration normalized to the peak value in the specimen, and $r = x\hat{x} + y\hat{y} + z\hat{z}$ is the position vector inside of the specimen. Here, we see that the SHG source term generating the SH scattered object field is proportional to the local normalized harmonophore concentration and local specimen orientation. These polarization densities lead to a second-harmonic field scattered by the incident field. We record the generated field as a coherent superposition of second-harmonic scattered within from the volume of the specimen illuminated by the fundamental field. We can record a hologram of this...
Fig. 1. Reconstructed field magnitude of 16 holograms of a 20 µm thick slice of corn seed, with varying excitation and analyzer polarizations. Rows vary excitation angle from 0 to 135° in 45° steps, from top to bottom; columns vary analyzer angle from 0 to 135° in 45° steps, from left to right. The colormap indicates total SHG intensity, with low intensity in black and high intensity in white.

field with the inclusion of a reference field [24]. An analyzer set at an angle $\psi$ with respect to the $x$-axis selects the polarization of the field incident on the camera as a superposition of the $x$ and $y$ SHG fields, as

$$E_{\text{SHG}}^x = E_{\text{SHG}}^x \cos(\psi) + E_{\text{SHG}}^y \sin(\psi).$$

(3)

We can also control the polarization of the illumination field, allowing us to set its polarization at some angle $\alpha$, again with respect to the $x$-axis. We record a set of holograms with varying illumination and analyzer angles, $\alpha = n\pi/4$ and $\psi = m\pi/4$, where $n$ and $m$ are both integer values between 0 and 3. Using standard holographic processing [24, 30–33] to back-propagate the holograms we can recover the field at any $z$ distance. We can write each field at this distance as

$$E_{m,n} = \gamma(r) F_{m,n} \exp(i\phi_{m,n}) \exp(i\phi_{m,n}).$$

With the normalized relative field magnitude given by

$$F_{m,n} = \cos\left(\frac{m\pi}{4}\right) \sin\left(\frac{n\pi}{4} + 2\theta\right) + \frac{1}{2} \sin\left(\frac{m\pi}{4}\right) \left[(1 + \rho) - (1 - \rho) \cos\left(\frac{n\pi}{2} + 2\theta\right)\right],$$

(4)

we can then write the total SHG intensity in the back-propagated plane as

$$I_n = \frac{1}{2} \sum_{m=1}^{4} |F_{m,n}|^2 = U + V \cos\left(\frac{n\pi}{4}\right) + W \cos(n\pi + 4\theta)$$

(5)

with $U = \frac{1}{8} (3\rho^2 + 2\rho + 7)$, $V = \frac{1}{8} (\rho^2 - 1)$, and $W = \frac{1}{8} (\rho^2 - 2\rho - 3)$ defined as in [17]. Solving for the parameters in terms of measured intensities, we arrive at equations for the three parameters in terms of the above-measured intensities,

$$U = \frac{1}{4} (I_0 + I_1 + I_2 + I_3), \quad V = \pm \frac{1}{2} \sqrt{\Delta I_{31}^2 + \Delta I_{02}^2}, \quad W = \frac{1}{4} (\Delta I_{01} + \Delta I_{23}) \frac{I_0^2 + I_3^2}{I_{02} - I_{31}^2}$$

(6)
where \( \Delta I_{ab} = I_a - I_b \). We can recover the sample-orientation angle \( \theta \) for each point in the reconstruction from these measurements as \( \tan(2\theta) = \Delta I_{31}/\Delta I_{02} \). As this angle estimate is derived from a ratio of intensities, it is insensitive to the local concentration of the harmonophore or the collection efficiency of the SH light. Making use of ratios eliminates local concentration and SHG intensity factors, allowing for extraction of local susceptibility parameters such as \( \rho \) and \( \theta \) without requiring knowledge of harmonophore concentration distributions in the specimen. Similarly, solutions for the value of the susceptibility ratio \( \rho \) can be formed from ratios of the any pair of the intensity terms \( U, V, \) and \( W \). While solutions of \( \rho \) should be independent of the calculation method, noise in the data present variations in the values extracted by each algorithm. Cross-checking multiple solutions for consistency of the extracted parameters validates the solutions of \( \rho(\mathbf{r}) \).

3. Experiment

The holography setup is a modification of our previously described system [24]. A Yb:KGW oscillator producing 340 fs pulses centered at 1027 nm is directed into a modified Mach-Zender interferometer. The reference field is generated in a 100 \( \mu \)m thick KH\(_2\)PO\(_4\) (KDP) crystal. The reference is separated from the fundamental using a dichroic filter, and directed to the camera through a spatial filter and adjustable time-delay arm. The remaining fundamental light passes through a half-wave plate for excitation polarization control and is focused onto the sample using a Meiji 4x 0.1 NA objective. The SHG signal is collected in the forward-scattered direction using a Zeiss Epiplan 50x 0.5 NA objective. The object and reference SHG beams are combined using a non-polarizing beam cube and pass through a wire-grid analyzing polarizer before being collected by an EM-CCD camera. This polarizer is rotated to measure different polarization projections on the camera.

We used as samples prepared slides of 20 \( \mu \)m thick sections of corn seed and canine tongue. The polarization of the horizontal linearly-polarized beam incident on the sample is rotated through 0\( ^\circ \), 45\( ^\circ \), 90\( ^\circ \), and 135\( ^\circ \). The resulting field is then joined with the reference, and the reference polarizer rotated through 0\( ^\circ \), 45\( ^\circ \), 90\( ^\circ \), and 135\( ^\circ \). This results in a collection of 16 holograms, each of which is back-propagated numerically to yield the field in the image plane. An example of the recovered field intensity is shown in Fig. 1, taken with a 20 \( \mu \)m thick slice of corn seed as the sample.

From these 16 fields, we calculate the four second-harmonic intensities, \( I_n \), by summing the intensity of each of the recovered field for all excitation polarizations for each analyzer polarization. From the four analyzer intensities, we can determine the value of the parameter \( U \). We can then calculate the other parameters, \( V \) and \( W \), from differences between the recovered intensities.

4. Results and discussion

From the set of 3D reconstructed SHG fields, we can obtain 3D image distributions of the total SHG scattered intensity (not modulated by the local projection of the incident polarization onto the symmetry axis of the organized tissue), the local SHG tensor symmetry axis orientation, \( \theta \), the SHG field phase, and the local value of \( \rho \). We will first concentrate on the first three quantities, and show 3D image data for SHG intensity, orientation, and phase at each \( z \)-plane in the sample. The total SHG intensity is computed from the data by \( I_{\text{SHG, tot}} = \frac{1}{4} \sum_{n=0}^{3} I_n = U \), with the local orientation computed through \( \theta = \frac{1}{2} \tan^{-1}(\Delta I_{31}/\Delta I_{02}) \). The local orientation is shown in Fig. 2(a-b), with lines superimposed on the total intensity above a SHG intensity reaching 14.5\% of the peak SHG intensity, i.e., for \( I_{\text{SHG, tot}} > 0.145 \times \max(I_{\text{SHG, tot}}) \). The phase difference between the SHG fields for horizontal excitation polarization and 45\( ^\circ \) analyzer polarization and vertical excitation and analyzer polarization, taken at the reference back-propagation plane, is...
Fig. 2. Frame from a movie showing reconstructed intensities of canine tongue (a, Media 1) and corn seed (b, Media 2), with polarization orientation indicated with white lines, showing reconstructions over a distance of 22 µm through the sample. Phase differences are shown for tongue (c) and corn seed (d), between projections \((n,m) = (1,2)\) and \((0,0)\).

shown in Fig. 2(c-d) for the threshold region as described above. The back propagation distance for these holograms is 58 µm. We display the SHG phase difference for only one pair of the sixteen polarization/excitation components, with the difference taken to remove the propagation phase, as we have found no significant variation in the phase behavior of the 16 polarization-isolated SHG holography terms. This suggests a lack of polarization dependence on SHG intensities, and that no birefringent phase matching effects are apparent in the specimens that we examined.

Determining the value of the susceptibility tensor ratio components is more challenging than the extraction of local orientation. We found solutions for \(\rho\) from each of the local intensity and concentration independent ratios of \(r_{VU} = V/U\), \(r_{WU} = W/U\), and \(r_{VW} = V/W\). As there is a sign ambiguity in \(V\), \(r_{VU}\) provides four solutions for \(\rho\), while \(r_{VW}\) and \(r_{WU}\) each provide two solutions, leading to a total of eight solutions for \(\rho\) in a single data set.

\[
\rho = \frac{-r_{VU} \pm 2\sqrt{4 + 4r_{VU} - 5r_{VU}^2}}{3r_{VU} + 4} \quad (7)
\]

\[
\rho = \frac{-r_{VU} \pm 2\sqrt{4 - 4r_{VU} - 5r_{VU}^2}}{3r_{VU} - 4} \quad (8)
\]

\[
\rho = \frac{-(1 + r_{WU}) \pm 2\sqrt{1 - 5r_{WU}^2}}{3r_{WU} - 1} \quad (9)
\]

\[
\rho = \frac{3r_{VW} \pm 4}{r_{VW}^2} \quad (10)
\]

The value of \(\rho\) is calculated for each pixel in the reconstructed SH field using the three different ratios (and the two possible roots, for the cases of \(r_{VU}\) and \(r_{WU}\)). From this array of values, we calculate the mean and standard deviation of all non-zero values contained in these retrieved \(\rho\) images. The multiple \(\rho\) solutions to provide a self-consistency check on the values of \(\rho\) obtained. In the data that we have analyzed to date, we found that the \(V-W\) ratio, \(r_{VW}\), provides the most consistent results, perhaps because it does not require the choice of a root in the solution and all values remain real. The only ambiguity is the sign of \(V\). For the tongue sample, as seen in Fig. 3, we determine four values for \(\rho\). From the \(V-W\) ratio, with \(V > 0\), we find \(\bar{\rho} = 3.4, \sigma = 1.3\). From the \(V-W\) ratio, with \(V < 0\), we determine \(\bar{\rho} = 2.9, \sigma = 1.4\). We can use the other ratios to verify this result. From the \(W-U\), positive root, we get \(\bar{\rho} = 3.4, \sigma = 1.3\).
while the negative root of the same ratio lacks sufficient non-zero values to yield a $\rho$ value. From the $V-U$ ratio, $V > 0$ with a positive $U$ root, we retrieve the value $\rho = 2.2$, $\sigma = 0.35$, while the $V < 0$ and both negative root solutions have so few non-zero datapoints as to not provide a solution. We see good agreement between the two values determined from the $V-W$ ratio, which is further verified by agreement with the value determined from the $W-U$ ratio. The divergent value, determined from the $V-U$ ratio, can then be given less weight in light of the good agreement of the other values.

5. Conclusion

We have demonstrated a polarization-based SHG holographic microscope, which we used to gather sample orientation and susceptibility ratios in three dimensions from unlabeled biological samples from a set of excitation and analyzer polarization-resolved SHG hologram images. From these data, we have numerically reconstructed three dimensional images of the total (not modulated by the incident field polarization) second-harmonic intensity scattered by the specimens. The holographic data provide access to the SH scattered field phase throughout the specimen volume. We found no polarization dependence on the phase images, which strongly indicates that there are no polarization-dependent phase variations, ruling out any birefringent phase matching effects on the SHG hologram image formation. The 3D distribution of sample orientation and the susceptibility ratio, $\rho$, of the local variations in $\chi^{(2)}$ tensor are also extracted from analysis of the PSHG hologram images. The methods presented have inherent consistency checks, and the agreement between the different methods has been examined as a validation metric. While a full analysis is presented here, it is clear that a sub-set of polarization-resolved SHG holograms can be recorded in order to extract 3D images of the total SHG intensity, orientation, optical phase, and relative susceptibility values. Given the ability to capture 3D images in a rapid time (each image could be acquired in less than a millisecond), this new PSHG holography opens the pathway for high-speed dynamical 3D PSHG imaging for studying biological processes, such as muscle mechanics with millisecond temporal resolution.

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