Effects of laser polarization and interface orientation in harmonic generation microscopy

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Abstract: We present systematic experimental investigations on the effects of laser polarization and interface orientation in second and third harmonic generation microscopy. We find that the laser polarization has no measurable effect on signal strength and resolution in third harmonic microscopy, while the second harmonic strongly depends upon the polarization direction of the driving laser. Moreover, we observe a strong effect of the interface orientation with respect to the laser beam direction—both in second and third harmonic generation. This affects the signal strength, as well as the obtained transversal and longitudinal resolution in microscopic imaging. As an (on the first glance) surprising feature, also surfaces parallel to the optical axis of the laser beam yield strong harmonic signal. This enables applications of harmonic microscopy in specific geometries. As an example we monitor the flow of immiscible microfluids in lateral cut by third harmonic microscopy.

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References and links
1. Introduction

In the past fifteen years coherent nonlinear microscopy (CNM) advanced towards a powerful tool for three-dimensional visualization of transparent samples (see e.g [1–3], and refs. therein). The basic concept of CNM is to drive frequency conversion processes in tightly focussed laser beams. The generated signal yields information on the material and geometric structure in the focal volume. By scanning the laser focus across the sample, we obtain a three-dimensional image. We note, that such nonlinear processes result in effective excitation volumes smaller than the diffraction-limited volumes of the fundamental field—which enables larger resolution. In contrast to multi-photon fluorescence microscopy [4], coherent nonlinear microscopy uses (coherent) frequency conversion processes rather than (incoherent) radiative decay. CNM requires no staining, marking or radiative quenching in the sample. Moreover, the applied off-resonant processes do not deposit energy in the sample [5], which may otherwise lead to heating and degeneration of the sample. Examples for CNM are imaging techniques involving second harmonic generation (SHG) [6], third harmonic generation (THG) [7] or coherent anti-Stokes Raman scattering (CARS) [8]. While CARS microscopy exploits resonances in the vibronic level structure of the sample [9], SHG and THG microscopy typically apply off-resonant frequency conversion processes. In the case of strongly focussed laser beams (which provide large signals and large resolution) SHG and THG appear only at interfaces [7, 10], while in the bulk medium the harmonics interfere destructively due to the Gouy phase shift [11]. We also note, that in contrast to THG, SHG occurs only at interfaces of non-centrosymmetrical media [5]. These features make harmonic generation microscopy a valuable instrument to image interfaces, even at the boundary of two transparent or even refractive index-matched species. Finally, microscopy by SHG and THG is rather easy to implement (e.g. in contrast to CARS only a single, sufficiently intense laser beam is required here). Thus, in recent years THG microscopy already found a larger number of demonstrations and applications, in particular to image biological systems [12–14].

Though the basic idea of harmonic microscopy is simple and straightforward, many important details of the imaging process are still a matter of debate and/or not fully understood. Thus, e.g. the question arises how the polarization direction of the driving laser beam or the orientation of the detected surface with respect to the laser beam affect a harmonic signal. Schwartz et al. already showed, that the polarization of the third harmonic signal is parallel to the linear polarization of the fundamental beam [15]. In the case of a circular polarized fundamental laser beam (and in media without birefringence), it was found that neither bulk material nor interfaces generate a third harmonic signal [16]. Masihzadeh et al. showed experimentally that the third harmonic intensity is independent of the fundamental laser polarization direction by observing surfaces oriented orthogonal to the beam propagation...
Numerical simulations for THG at surfaces oriented parallel to the laser beam direction predicted a considerable dependence of THG vs. polarization direction [18, 19]—though different authors gave different values and signs of the predicted changes. To clarify this point, we require systematic, experimental investigations on the dependence of harmonic signals vs. the laser polarization direction at surfaces oriented parallel to the laser beam. Moreover, so far there are only numerical simulations on the effect of surface orientation in THG microscopy [19]. This dependence is very important for applications—as specific surface orientations may not yield harmonic signal, i.e. may not permit imaging of complex structures or specific geometries. Thus, also here we require detailed experimental studies.

In the following, we present systematic experimental investigations with regard to the effects of laser polarization and interface orientation in SHG and THG microscopy. We performed measurements to obtain clear and sharp three-dimensional and two-dimensional images of an appropriate test object. Moreover, we apply our findings to image a microfluidic system in lateral cut by THG microscopy.

2. Experimental implementation

Figure 1 depicts the schematic setup of our nonlinear microscope. We apply a femtosecond Titanium:Sapphire laser oscillator (Spectra-Physics, Tsunami), pumped by a continuous wave Nd:YVO₄ laser (Coherent, Verdi G7). The laser setup provides a train of ultra-short pulses at center wavelength of 810 nm, average output power of 1W, pulse duration of 60 fs (FWHM of intensity), and repetition rate of 82 MHz. The laser beam is focused by a microscope objective (Zeiss, 46 04 08) with a numerical aperture of 0.22 and focal length of 16 mm into the sample. The second harmonic (at 405 nm) and third harmonic (at 270 nm) generated in the sample at the laser focus are collimated by a condenser (NA = 0.67), separated by dichroic mirrors (DM) and interference filters (IF), detected on photo-multiplier tubes (PMT) and processed in a computer. A galvano scanner enables two-dimensional scanning of the laser focus in the xy-plane of the sample, i.e. perpendicular to the optical axis of the laser beam. The microscope objective is mounted on a translation stage, which permits scanning of the laser focus in the z-direction, i.e. parallel to the optical axis of the laser beam. Thus, we are able to obtain three-dimensional images with a spatial resolution in the micrometer range.

To systematically monitor the effects of laser polarization and interface orientation in harmonic microscopy we use a fused silica capillary (Qsil, DA1.8DI0.15) in a refractive index-matched fluid (Cargille, 50350) as a test object. The capillary has an inner diameter of 200 µm and an outer diameter of 1.8 mm. Such a transparent object in an also transparent and even index-matched fluid is totally invisible by conventional (i.e. linear optical) microscopy. Thus, the setup already exhibits a nice demonstration of the potential and advantages of
harmonic microscopy. Moreover, the cylindrical geometry of the capillary possesses a continuous variation of the interface orientation and permits systematic observation of orientation effects in harmonic generation microscopy. We note, that Barille et al. demonstrated the basic feasibility of third harmonic microscopy to image cylindrical objects (optical fibers made of fused silica) [20]. However, Barille et al. did not perform any systematic measurements on the dependence of the THG signal with regard to polarization directions and interface orientations—which is the aim of the following work.

3. Experimental results

In the following we present and analyze images of the capillary taken by harmonic microscopy at appropriate alignments of the capillary symmetry axis with regard to the laser beam axis. This reveals the dependence of the harmonic signal (rp. the obtained microscopic image) vs. polarization direction or interface orientation.

3.1 Dependence of harmonic generation upon laser polarization

In a first experiment we align the symmetry axis of the capillary parallel to the optical axis of the laser beam, i.e. in z-direction (see inset in Fig. 1). The major part of the capillary surface is oriented parallel to the optical laser beam axis. Let us call this surface geometry “longitudinal”. The laser beam is linearly polarized, either in x-direction or in y-direction. We take three-dimensional images of the test object by SHG and THG microscopy. From the three-dimensional images we deduce cuts in the xy-plane at fixed z-position, i.e. yielding a two-dimensional lateral cut of the capillary.

Figure 2 shows images of the capillary (i.e. the interface between the inner diameter of the capillary and the index-matched fluid in the center), experimentally obtained by scanning THG microscopy. For a first overview, the right side of Fig. 2 shows the measured, full three-dimensional THG image of the inside of the capillary. We get a very clear and sharp, three-dimensional image of the interface. We note, that for better visibility we reduced the available information depth and do not show the variation of the signal intensity along the capillary surface in this three-dimensional image. This information is depicted in the two-dimensional
lateral cuts on the left side of Fig. 2, which show the variation of the detected THG signal in the xy-plane. Here color coding indicates the measured THG intensity. We performed two experimental runs of this measurement, either for horizontal or vertical laser polarization—as indicated by the orange arrows in Fig. 2. Also in these cuts in the xy-plane we observe very clear and sharp images. Already on the first glance the images for the two polarizations look very similar with regard to the THG intensity. For any arbitrary point on the capillary surface it makes no large difference, whether the incident laser beam is horizontally or vertically polarized.

This finding becomes very obvious, if we plot the difference signal of the two images for horizontal and vertical polarization from Fig. 2. The resulting contour plot of the difference image in the left side of Fig. 3 shows a very faint structure, i.e. a difference signal very close to zero. The same holds true, when we use an alternative plot type for the relative difference signal and depict it vs. the angle $\phi$ along the capillary surface (see right graph in Fig. 3, for the definition of the angle $\phi$ see Fig. 2). Besides some statistical signal fluctuations, we do not see an effect of the polarization direction upon the THG intensity at a longitudinal interface. Taking our measurement accuracy into account, we estimate the effect of the polarization direction upon the THG intensity to be below 5% (e.g. if we change from horizontal to vertical polarization).

![Two-dimensional contour plot showing the difference signal of the two images for horizontal and vertical polarization from Fig. 2. Color coding along the capillary surface indicates the intensity of the THG difference signal. The image consists of 1000 x 1000 data points. (right) Relative change of the THG difference signal versus the angle $\phi$ along the capillary surface. Due to the intrinsic symmetry of the test object and image, we plot data for $\phi = -\pi/2$ to $\phi = +\pi/2$ only.](image)

Fig. 3. (left) Two-dimensional contour plot, showing the difference signal of the two images for horizontal and vertical polarization from Fig. 2. Color coding along the capillary surface indicates the intensity of the THG difference signal. The image consists of 1000 x 1000 data points. (right) Relative change of the THG difference signal versus the angle $\phi$ along the capillary surface. Due to the intrinsic symmetry of the test object and image, we plot data for $\phi = -\pi/2$ to $\phi = +\pi/2$ only.

Thus, the data in Figs. 2 and 3 clearly indicate a negligible effect of the polarization direction upon the third harmonic yield, i.e. the quality of the image in THG microscopy. We note, that these experimental findings contradict previous numerical simulations. Olivier et al. predicted an increasing THG intensity of 35% by rotating the polarization from parallel to perpendicular to an interface [18]. In similar simulations, Cheng et al. surprisingly calculated a decrease of 20% in the THG intensity by rotating the polarization from parallel to perpendicular [19]. Thus, the simulations contradicted each other—but both yielded a considerable effect of the polarization upon the THG signal. We note, that the authors of [18, 19] did not give a physical explanation for their numerical findings. Our experimental data clearly show no effect of the polarization upon THG. Our results agree well with the intuitive picture of THG in a medium with isotropic third order susceptibility (e.g. as fused silica [21]). As the medium is isotropic, there should be no reason for a polarization dependence in THG.
In addition to THG microscopy, we also took images of the capillary (i.e. the interface between the inner diameter and the index-matched fluid in the center) by SHG microscopy. Figure 4 shows the measured SHG difference signal, i.e. taking the difference of SHG images at perpendicular polarizations. We plot the difference image (see left graph in Fig. 4) and alternatively the relative variation of the difference signal with the angle $\phi$ (see right graph in Fig. 4). As both graphs show, we observe a very large variation (i.e. up to 300%) in the SHG intensity, when we change the laser polarization from horizontal to vertical. Thus, SHG imaging depends strongly upon the choice of the laser polarization—in contrast to THG imaging. The reason for the strong polarization dependence of the SHG signal is a non-vanishing, an-isotropic second order susceptibility, which may occur even in an amorphous material such as fused silica [22]. Due to this second order an-isotropy the SHG yield depends upon the laser polarization. Thus, an appropriate choice in laser polarization permits better contrast in SHG microscopy—while for THG the polarization does not matter.

Fig. 4. (left) Two-dimensional contour plot, showing the difference SHG signal of the two images for horizontal and vertical polarization. Color coding along the capillary surface indicates the intensity of the SHG difference signal. The image consists of 1000 × 1000 data points. An animation of the full three-dimensional SHG image (not depicted here) is available at the online link Media 2. (right) Relative change of the SHG difference signal versus the angle $\phi$ along the capillary surface. Due to the intrinsic symmetry of the test object and image, we plot data for $-\pi/2 < \phi < +\pi/2$ only.

### 3.2 Dependence of harmonic generation upon interface orientation

To investigate the effect of the interface orientation upon the harmonic yield, we change now the experimental geometry and align the symmetry axis of the fused silica capillary perpendicular to the propagation direction of the incoming laser beam. In our coordinate system (see also Fig. 1) the $z$-direction is still defined by the optical axis of the laser beam. The laser polarization is set parallel to the $x$-axis, though also any other choice of the linear polarization direction would make no difference (see sect. 3.1 above). The symmetry axis of the capillary is oriented along the $y$-axis. In this setup, the surface of the capillary continuously varies from an orientation perpendicular to the laser beam axis (“transversal” geometry) towards an orientation parallel to the laser beam axis (“longitudinal” geometry). Thus, by taking a lateral cut image of the capillary in $xz$-plane we directly image the effect of the interface orientation upon the harmonic yield.

Figure 5 shows a lateral cut ($xz$-plane) of the capillary, recorded by THG microscopy. To indicate the position on the capillary surface (rp. the interface orientation), we introduce the angle $\theta$ with respect to the $z$-axis. The ring-like image clearly mirrors the interface between
the inner diameter of the capillary and the refractive index-matched fluid in the center. The image reveals a quite strong effect of the interface orientation upon THG yield and spatial resolution. For better quantitative analysis, Fig. 5 also depicts intensity profiles along horizontal and vertical pathways through the image.

Most obviously from the THG image, the spatial resolution (i.e. the “width” of the THG signal) is much better for longitudinal interface orientation (i.e. parallel to the laser beam axis) compared to transversal interface orientation (i.e. perpendicular to the laser beam axis). In particular, the intensity profile at parallel orientation (e.g. at $\theta = \pi/2$) yields a width (FWHM) of 2 $\mu$m, while the intensity profile at transversal orientation (e.g. at $\theta = \pi$) yields a width of 29 $\mu$m. Thus, the difference in the resolution is approximately a factor of 14. We attribute this difference mainly due to the fact, that the extension of the focus in propagation z-direction (e.g. defined by twice the Rayleigh length) is larger than the extension of the focus in the xy-plane. Thus, scanning in the xy-plane will yield better resolution than scanning in z-direction. The optical specifications of the microscope objective in our setup confirm this interpretation: We expect an aspect ratio of approximately 7 in the focal volume, generated by our microscope objective under ideal conditions, e.g. in a sample without refraction. Intrinsic imperfections in the optics and refraction at the entrance of the sample will further increase the aspect ratio towards the observed ratio.

![THG microscopy of the capillary in lateral cut in the xz-plane. The orientation of the capillary is along the y-axis, i.e. perpendicular to the optical laser beam axis. The image consists of 920 × 1016 data points. Color coding along the capillary surface indicates the intensity of the THG signal in arbitrary units. The slightly larger intensities in positive x-direction are due to rather typical alignment variations in a mirror-scanning setup. On the upper and right side of the image we add traces of the THG intensity along horizontal and vertical pathways through the image (indicated by the yellow lines in the image).](image)

We consider now the observed THG intensity at different interface orientations. We note, that THG from interfaces in longitudinal orientation were observed before [7], but has not yet been investigated systematically. As our measured intensity profiles show, the THG intensity at longitudinal interface orientation (i.e. parallel to the laser beam axis) is a factor of approximately 1.5 larger compared to the THG yield at transversal interface orientation (i.e. perpendicular to the laser beam axis). On the first glance, this seems surprising. The intuitive “simple man’s explanation” of THG microscopy typically requires a transversal interface [7] to break the isotropy of the sample and lead to non-vanishing destructive interference of THG in the laser focus. In this very simplified picture, an interface in longitudinal orientation is expected to yield no THG signal at all. As numerical simulations by Cheng et al. beyond the
intuitive picture showed, we may expect some THG signal also at interfaces in longitudinal orientation [19]. The simulations predicted, that the signal at transversal interfaces is expected to be larger compared to the signal from longitudinal interfaces—which also agrees with the simplified picture of THG. Our experimental data contradict this numerical finding and the simple picture. Longitudinal interfaces yield larger signal compared to transversal interfaces. We believe, that the aspect ratio of the focal volume plays an important role here. At interfaces parallel to the laser propagation direction, the geometrical overlap of focal volume and interface is much larger than at interfaces perpendicular to the laser beam direction. Thus, the region which effectively emits the third harmonic is much larger at longitudinal interfaces—a stronger THG signal in the image. We note, that we observed a similar dependence of spatial resolution and harmonic yield vs. interface orientation also in SHG microscopy. As the results are similar to THG microscopy, we do not show the SHG images or discuss details here.

The possibility to obtain strong THG signal at longitudinal interfaces (as discussed above) is a quite important finding – as it enables particular applications of harmonic microscopy. As a demonstration example, we took THG images of a specific microfluidic system in a substrate of glass and polydimethylsiloxane (PDMS), see Fig. 6. No staining, marking, or use of quenching effects and resonances is required to image the interfaces by harmonic microscopy. The sample is a device of three transparent liquids flowing parallel in y-direction without mixing. In our example, a thin layer of dextran is flowing inside two broader layers of polyethylene-glycole (PEG). The two liquid-liquid interfaces between dextran and PEG are oriented in z-direction. Such a specific microfluidic device enables, e.g. size-dependant detachment of DNA molecules at liquid-liquid interfaces [23]. The task is to determine position and extension of the transparent liquid-liquid dextran-PEG interfaces, i.e. the “sheath flow” [24]. The microfluidic chip is rather thin in z-direction, but thick in x-direction. Thus, imaging by harmonic microscopy is only possible with the laser propagating in z-direction, i.e. parallel to the liquid-liquid interface. Nevertheless, the image taken by THG microscopy clearly reveals the two longitudinal liquid-liquid dextran-PEG interfaces, i.e. the vertical phase boundaries of the “sheath flow”. The latter have a width of only a few microns – which is very well resolved in the image. We note, that the image also reveals the interfaces between the flows and the surrounding solid substrate material (as expected).

![Fig. 6. THG microscopy of a microfluidic system of three liquids (PEG, Dextran and PEG) flowing in y-direction. The obtained resolution is 1 × 1 µm². The underexposure in the corners of the microfluidic channels is due to shadowing of THG signal due to the channel geometry.](image)

5. Conclusion

We presented and discussed systematic experimental investigations with regard to the effect of laser polarization and interface orientation in harmonic generation microscopy, i.e. SHG and THG microscopy. As a test object, we used a fused silica capillary in an index-matched liquid—which would be totally invisible in conventional (linear) microscopy. The obtained high-resolution three-dimensional images and two-dimensional lateral cuts of the test object in
SHG and THG microscopy reveal the following characteristic features: (1) The direction of the laser polarization plays no role for strength and resolution in the image, taken by THG. This contradicts previous numerical simulations – but agrees well with the intuitive picture of THG at interfaces. (2) The direction of the laser polarization strongly affects SHG microscopy, i.e. leads to a large modulation of the signal strength. This is due to some residual second order an-isotropy even in an amorphous material such as fused silica. (3) The interface orientation affects signal strength and spatial resolution, both in THG as well as SHG microscopy. As an (on the first glance) surprising feature also, interfaces oriented parallel to the laser beam direction yield strong harmonic signal. This is due to the larger “effective” area responsible for harmonic emission in this geometry. The experimental data contradict previous numerical simulations—which obviously were incomplete. Finally we demonstrated the consequences of the latter finding by imaging a microfluidic flow in lateral cut, obtaining a high-resolution image of a “sheath flow”.

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