Lens-less surface second harmonic imaging

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Abstract: Lens-less surface second harmonic generation imaging (SSHGI) is used to image an SHG active molecule, (S)-( + )-1,1′-bi-2-naphthol (SBN), incorporated into a lipid bilayer patterned with the 1951 United States Air Force resolution test target. Data show the coherent plane-wave nature of SHG allows direct imaging without the aid of a lens system. Lens-less SSHGI readily resolves line-widths as small as 223 μm at an object-image distance of 7.6 cm and line-widths of 397 μm at distances as far as 30 cm. Lens-less SSHGI simplifies the detection method, raises photon collection efficiency, and expands the field-of-view. These advantages allow greater throughput and make lens-less SSHGI a potentially valuable detection method for biosensors and medical diagnostics.

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References and links

1. P. A. Franken, A. E. Hill, C. W. Peters, and G. Weinreich, “Generation of optical harmonics,” Phys. Rev. Lett. 7(4), 118–119 (1961).
2. N. Bloembergen, R. K. Chang, S. S. Jha, and C. H. Lee, “Optical second-harmonic generation in reflection from media with inversion symmetry,” Phys. Rev. 174(3), 813–822 (1968).
3. Y. R. Shen, “Surface properties probed by second-harmonic and sum-frequency generation,” Nature 337(6207), 519–525 (1989).
4. T. F. Heinz, M. M. T. Loy, and W. A. Thompson, “Study of Si(111) surfaces by optical second-harmonic generation: reconstruction and surface phase transformation,” Phys. Rev. Lett. 54(1), 63–66 (1985).
5. D. Heskett, K. J. Song, A. Burns, E. W. Plummer, and H. L. Dai, “Coverage dependent phase transition of pyridine on silver(110) observed by second harmonic generation,” J. Chem. Phys. 85(12), 7490–7492 (1986).
6. R. M. Corn, M. D. Levenson, and M. R. Philpott, “The potential dependence of surface plasmon-enhanced second-harmonic generation at thin film silver electrodes,” Chem. Phys. Lett. 106(1–2), 30–35 (1984).
7. J. S. Salafsky, “Second-harmonic generation as a probe of conformational change in molecules,” Chem. Phys. Lett. 381(5-6), 705–709 (2003).
8. J. M. Hicks and T. Petralli-Mallow, “Nonlinear optics of chiral surface systems,” Appl. Phys. B 68(3), 589–593 (1999).
9. J. S. Salafsky and K. B. Eisenthal, “Protein adsorption at interfaces detected by second harmonic generation,” J. Phys. Chem. B 104(32), 7752–7755 (2000).
10. R. Hellwarth and P. Christensen, “Nonlinear optical microscopic examination of structure in polycrystalline ZnSe,” Opt. Commun. 12(3), 318–322 (1974).
11. Y. R. Shen, “Surface second harmonic generation: a new technique for surface studies,” Annu. Rev. Mater. Sci. 16(1), 69–86 (1986).
12. A. Moret, G. Ruffy, A. Del Guerzo, C. Belin, M. Dussauze, V. Rodriguez, and J.-M. Vincent, “Chemisorption of fluorous copper(II)-carboxylate complexes on SiO2 surfaces: versatile binding layers applied to the preparation of porphyrin monolayers,” Chem. Commun. (Camb.) 46(15), 2617–2619 (2010).
13. M. Iwamoto and T. Manaka, “Probing and modeling of carrier motion in organic devices by optical second harmonic generation,” Thin Solid Films 519(3), 961–963 (2010).
14. M. A. Kriech and J. C. Conboy, “Imaging chirality with surface second harmonic generation microscopy,” J. Am. Chem. Soc. 127(9), 2834–2835 (2005).
15. P. Campagnola, “Second harmonic generation imaging microscopy: applications to diseases diagnostics,” Anal. Chem. 83(9), 3224–3231 (2011).
16. C.-S. Liao, Z.-Y. Zhuo, J.-Y. Yu, Y.-Y. Tseng, S.-W. Chu, S.-F. Yu, and P.-H. G. Chao, “Decrimping: The first stage of collagen thermal denaturation unraveled by in situ second-harmonic-generation imaging,” Appl. Phys. Lett. 98(15), 153703 (2011).
1. Introduction

Second harmonic generation (SHG) was first demonstrated from a crystalline quartz sample in 1961 by Franken and associates [1]. Taking advantage of the observation that SHG is only produced in non-centrosymmetric media, Bloembergen and associates demonstrated that interfaces can also produce optical SHG [2]. Bloembergen’s discovery of the ability of SHG to probe chemical and physical properties of interfaces led to the wide use of SHG as a valuable surface science technique spanning several disciplines, such as physics, chemistry, biology, and material science [3–7]. As a surface science technique, SHG has been used to examine various properties of interfaces, such as the surface structures of metals [5], the structural symmetry of a semiconductor’s surface layer [4], the effective surface charge density on an electrode due to adsorption of ions [6], the conformational changes of biomolecules tethered to a surface [7], and protein adsorption at liquid/solid interfaces [8, 9]. SHG has proven to be a valuable spectroscopic technique; however, it was not until Hellwarth and Christensen combined SHG with an optical microscope that SHG was first used as an imaging technique [10]. Implementing SHG as an imaging technique offered many benefits to existing methods, such as direct imaging without a fluorescent label, submonolayer sensitivity, non-destructive, and time-resolution capabilities [11]. SHG has been used as a surface sensitive imaging technique to visualize the uniformity of the interfacial region of a metalloporphyrin film [12], the carrier motion at interfaces of organic devices [13], and the chirality of surface immobilized small molecules [14]. Although SHG imaging has been extensively employed in the field of biological tissue imaging [15, 16], these studies have utilized the bulk structural symmetry of proteins in tissue or cells to generate the SH signal. The reports of surface SHG imaging (SSHGI) of biological interfaces are primarily limited to studies that probe SH-active dye molecules to monitor membrane potential [17, 18], individual liposomes [19], and kinetic transport [20, 21]. In efforts to reach single molecule detection, membrane proteins labeled with a gold nanoparticle have also been examined using...
SHG imaging [22]. Recently, a label-free surface SHG imaging study in our lab probed drug-lipid interactions at a liquid/solid interface in a high-throughput manner [23].

All of the aforementioned studies convey the versatility of SSHG and its effectiveness in imaging, but like most other imaging techniques the previous SSHG imaging studies utilized a lens system to reconstruct the surface image from the emitted SHG light. In this paper, we demonstrate that the long coherence length and plane-wave nature of surface SHG minimizes diffraction and therefore, makes lens-less SSHG imaging possible. Admittedly the idea of removing the lens from an imaging system is not new. Electron microscopy without lenses was demonstrated in 1948 by Gabor et al when he used the recorded image of the Fresnel diffraction pattern from an object to reconstruct the image of the object [24]; however, this lens-less holographic imaging technique requires algorithms to reconstruct the image of the object [25]. Although advances in lens-less holographic microscopy designed to eliminate the need for propagation algorithms, such as wavelength multiplexing [25], have been demonstrated, a reconstruction and decoding stage is still necessary. Thus, despite overcoming some of the shortcomings of lenses, holographic microscopy inherently only allows for indirect imaging. If an optical imaging system could directly image the amplitude distribution from a surface without incorporation of an objective lens, it would greatly simplify the imaging process while still avoiding the limitations of lenses. Ideally, the incorporation of an objective lens in an optical imaging systems should only be necessary to resolve an image if diffraction and scattering effects cause divergence of the beam as it travels through space [26]. When SSHG is produced with the use of a collimated light source with limited divergence, a collimated coherent plane-wave is produced. Theoretically, under these conditions, the need for an objective lens could be eliminated.

In order to test the feasibility of lens-less SSHGI, images of various sized lipid bilayers containing an SH active molecule were obtained at several distances. According to optical beam propagation theory, the transversal amplitude distribution or intensity is dependent on the propagation distance and the initial beam width [27]. In SSHGI, each object behaves as a local emitter of light and produces its own propagating beam where the object size can be taken as the initial beam width. Since the object size is related to the initial beam width, it will influence how rapidly the transversal amplitude distribution spreads as the beam propagates through space [27]. Therefore, the behavior of the SHG propagating beam from each lens-less image can be characterized by analyzing the imaged object beam width as a function of distance using optical beam propagation theory. To further demonstrate that minimal divergence is required for imaging without an objective lens a comparison to an identically set up lens-less fluorescence imaging study where the generated wave-front is instantaneously spherical was also conducted.

To the best of our knowledge, this idea of imaging without a lens using surface SHG has never been demonstrated and more significantly lens-less imaging has never before been demonstrated in the ultra-violet wavelength range. The lens-less SSHG imaging method presented in this paper is considerably simpler than lens-less holographic microscopy. No reconstruction of the object is necessary as the intrinsic second harmonic amplitude distribution of the object can be directly imaged, thus only requiring a detector. Additionally, the removal of the microscope and objective lens allows more light to be collected, increasing the overall photon collection efficiency as it eliminates the amount of light loss through a microscope and aperture. Furthermore, without the objective lens the field of view or detection area is no longer limited by the magnification of the objective lens, but rather the size of the beam or illumination area. The larger detection area possible with lens-less SSHG imaging could increase throughput significantly relative to imaging with an objective lens, which in the field of biosensors and medical diagnostics would be advantageous. On a more fundamental level, the lens-less SSHG imaging method presented in this paper illustrates the differences between coherent and incoherent imaging. Coherent plane-wave imaging was thoroughly investigated by using Gaussian beam propagation theory to describe the effect of
the object size and the detector-sample distance on image formation and diffraction. Gaussian beam propagation theory was also shown to accurately describe the observed lens-less SSHG images.

2. Theory of lens-less SHG imaging

In a typical imaging system, diffraction caused by the transversal spreading of the light source leads to an unavoidable increase in the imaged object beam width as the propagation distance increases [26]. For this reason focusing elements, such as a lens, are used to reconstruct the image at some distance from the object [26]. However, if there were minimal transversal spreading and therefore negligible change in the imaged beam width, images could be resolved without the use of a lens. Since transversal spreading results when the electromagnetic waves emitted from an object are not parallel, not in phase, or not planar, an imaging source that produces parallel waves with constant phase and frequency is needed if one wishes to eliminate the need for an objective lens. In other words, lens-less imaging is only possible if the imaged light source is a collimated (parallel), coherent (in phase) plane wave (constant phase and frequency). Surface SHG is a technique, which under the proper conditions can generate a plane-wave that is both coherent and collimated over a long distance providing the possibility for lens-less imaging.

The SH response from the surface is produced when two light waves of the same frequency are spatially and temporally overlapped at the surface [28]. The resulting SHG emission can be described by Eq. (1):

\[ I_{\text{SH}} \propto \left| f_{\text{SH}} f_{\text{f}}^2 \chi^{(2)} \right|^2 \]  

(1)

where \( \chi^{(2)} \) is the nonlinear susceptibility tensor and \( f_{\text{f}} \) and \( f_{\text{w}} \) are the Fresnel coefficients for the SH and incident fields. \( \chi^{(2)} \) dictates the interaction of the applied electric fields \( E_{\text{i}}(\omega) \) and \( E_{\text{f}}(\omega) \), and the resulting SH field at the surface. Since SHG is a second-order nonlinear process in which two photons of the same frequency are spatially and temporally overlapped (no relative phase change) to generate a third photon of twice that frequency (narrow frequency distribution), there exists a near constant phase and frequency in the output and thus coherent plane waves are generated. The two incoming electromagnetic waves, \( A \) and \( B \), with the same frequency, \( \omega_0 \), can be written as shown in Eqs. (2) and (3), respectively [29, 30]:

\[ f(x, t) = A \exp \left[ i(k(x)\omega - \omega_0 + \delta_\text{A}(t)) \right] \]  

(2)

\[ f(x, t) = B \exp \left[ i(k(x)\omega - \omega_0 + \delta_\text{B}(t)) \right] \]  

(3)

where \( k \) is the propagation vector, \( A \) and \( B \) are the amplitude of waves travelling some distance \( x \) over time \( t \), and \( \delta_\text{A} \) and \( \delta_\text{B} \) are the phase factors for wave \( A \) and \( B \), respectively. Since the frequency distribution of waves \( A \) and \( B \) is narrow due to the monochromatic incident light, then on average \( \delta_\text{A} \) and \( \delta_\text{B} \) do not change significantly for a given period. Additionally, since the two electromagnetic waves interact in such a way that there is no relative phase difference, \( \delta(t) = \delta_\text{A}(t) - \delta_\text{B}(t) = 0 \), the generated SHG wave can be described by Eq. (4):

\[ f(x, t) = E(2\omega) \exp \left[ i(k_{\text{SH}}(2\omega)x - 2\omega_0) \right] \]  

(4)

Due to the incident beams having both a narrow frequency distribution and negligible phase difference there is minimal constructive and destructive interference [29], producing a SHG output that has a constant relative phase and is therefore coherent. The coherence of the electromagnetic waves creates infinite parallel planes of uniform amplitude distribution.
(constant frequency and phase), meaning that the generated surface SHG wave has a planar wave-front. However, the above derived expressions are only valid under the assumption that the incident waves are plane-waves where the sum of the propagation vectors for each of the incident beams are parallel and thus propagate in a single direction. If the incident beams were focused on the sample their wave-fronts would be near radial due to the large distribution of k vectors. Consequently, in order to assure the incident beams are plane-waves having a single propagation direction, they must be highly collimated.

In the counter-propagating SHG geometry used here (shown in Fig. 1) where the two incident light waves approach the sample from opposing directions there is a change of sign of the x component of the propagation vector, which due to the conservation of momentum, the generated SH signal is produced normal to the surface. Generating the SHG output normal to the surface further eliminates any distortion to the wave-front caused by emission at an angle, which helps produce a more planar wave-front.

Under the conditions described above, surface SHG generates both a collimated and coherent electromagnetic plane-wave where transversal spreading is negligible over a significant distance. In principle, imaging without an objective lens should be possible. However, since the generated wave is not stationary it is important to consider the behavior of the plane-wave as it propagates through space. A plane-wave of constant phase and frequency can persist and propagate along a given direction (z axis) if the plane (x-y plane) is also infinite perpendicular to the propagation axis. From an imaging perspective, the objects which generate the SH field will be of finite dimension, thus generation of an infinite plane-wave is not possible [31]. However, if the object dimension is a sufficiently large number of wavelengths there will be a slow rate of transversal spreading and the plane-wave approximation can be made. Additionally, since SHG produces relatively narrow and highly collimated beams which propagate relatively parallel to the optical axis, the paraxial approximation can also be used [27]. Assuming the SHG output from each object has an ideal Gaussian intensity profile (TEM00 Gaussian mode) the SHG beam can be described by solutions to the paraxial wave equation for a coherent plane-wave with a Gaussian intensity profile. The solutions to the wave equation under these conditions lead to two important beam parameters, the object width (w(z)) and the radius of curvature (R(z)), which represent the expansion of the beam width with propagation distance and the curvature of the phase front, respectively. (shown graphically in Fig. 2(A) and 2(B)) [32].
Equation (5) describes the variation of the image width at any plane at some distance \( z \) perpendicular to the propagation axis [27],

\[
w(z) = w_0 \sqrt{1 + \left( \frac{\Delta z}{\pi w_0^2} \right)}.
\]  

(5)

where \( w_0 \) is the initial object width and \( \lambda \) is the wavelength. From Eq. (5) it can be seen that the divergence of the beam or spreading of the image width is not only dependent on the propagation distance, but is also highly dependent on the initial object width where small objects lead to a much more rapid transversal spreading of the beam and a greater deviation from an ideal plane-wave. The curvature of the phase front of the beam at any distance \( z \) can be determined by,

\[
R(z) = z \left[ 1 + \left( \frac{\pi w_0^2}{\lambda z} \right) \right].
\]  

(6)

Equation (6) demonstrates that as the distance from the object increases \( R(z) \) becomes larger and significant curvature of the wave-front occurs. At sufficiently large distances from the object the beam has a spherical wave-front; this drastic deviation from a plane wave-front causes classic diffraction. The distance at which the wave-front is no longer planar, or collimated, occurs when \( w(z) \) has increased by \( \sqrt{2}w_0 \) [27]. This distance is referred to as the Rayleigh range or confocal distance, \( z_r \), and can be determined by setting Eq. (5) equal to \( \sqrt{2}w_0 \) and solving for \( z \) to give [27],

\[
z = z_r = \frac{\pi w_0^2}{\lambda}.
\]  

(7)

As seen in Eq. (7), the smaller the initial object width, \( w_0 \), the shorter the collimated region [26]. Past this collimated region where the wave-front is significantly curved, diffraction will be evident and imaging without a lens system would not be possible. Consequently, the ability to image without a lens is dependent on both the initial object size and the object-image distance.

In order to test the hypothesis that lens-less SSHGI is possible without a lens system, SHG images of a lipid bilayer containing an SH active molecule, (S)-(-)-1,1′-bi-2-naphthol (SBN, 99%), patterned into various sized line-widths using the United States Air Force (USAF) test pattern were obtained at several object-image distances. Imaging different sized objects at different distances provides an efficient means of evaluating lens-less SSHG imaging using Gaussian beam propagation theory.
3. Pattern fabrication and measurements

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine-cap-DOPE) were obtained from Avanti Polar Lipids and used as received. SBN was obtained from Sigma Aldrich and used as received. All water used in the experiments was obtained from a Nanopure™ Infinity Ultrapure water system with a minimum resistivity of 18.2 MΩ·cm. Phosphate buffered saline (PBS) was made from 50 mM Na2HPO4·7H2O and 100 mM NaCl in water and adjusted to a pH of 7.4 using NaOH. SBN was dissolved in PBS pH 7.4 to the desired working concentration (55 nM). Planar supported lipid bilayers (PSLBs) were prepared on custom manufactured full spectrum grade (IR/UV) fused silica prisms (Almaz Optics). The prisms were cleaned by immersion in a solution of 70% sulfuric acid and 30% hydrogen peroxide overnight. (CAUTION: this solution is a strong oxidant and reacts violently with organic solvents. Extreme caution must be taken when handling the solution). Prior to use, the prisms were rinsed thoroughly with water and cleaned with Ar plasma (Harrick Scientific Plasma Cleaner/Sterilizer) for 3 minutes.

All lipids were dissolved in chloroform, followed by evaporation under a gentle stream of N2(g) and then vacuum dried overnight to remove residual solvents. Small unilamellar vesicle (SUV) solutions were formed by re-suspending the dried lipids in PBS to a concentration of 1 mg/mL followed by vortexing and bath sonication for 10 - 30 minutes until the solutions became clear.

The prism used as the PSLB substrate was mounted in a custom built Teflon manifold (volume of 1 mL). A PSLB was formed on the silica prism by vesicle fusion, which involved incubating the surface with the SUV solution for 20 minutes at room temperature. The manifold was then flushed with PBS to remove any free lipid solution. The 1951 (USAF) positive resolution test target (chrome pattern on UV transparent glass) purchased from Edmund Optics was placed on top of the PSLB gently and taped down allowing only a small water layer to remain between the PSLB and test target. The prism with the resolution test target was placed in an ultra-violet ozone (UVO) cleaner (Jelight Co.) with a low pressure mercury vapor grid lamp for 13 minutes. The PSLB not covered by the chrome pattern was etched by the UV light to form the positive resolution test pattern [33].

After formation of the resolution test pattern, the prism was mounted in a custom built flowcell (volume of 0.4 mL) under Ultrapure water. SBN was then injected into the flowcell and allowed to adsorb to the patterned DOPC bilayer. SBN was chosen because it resonantly enhances the SH emission and preferentially intercalates into the DOPC bilayer [14]. giving rise to a large SH signal where there is a formed bilayer and negligible nonspecific adsorption to the silica prism where the bilayer has been etched away. After adsorption of the SBN into the bilayer, the flowcell was then flushed with PBS to remove any unbound SBN before images were taken.

Counter-propagating SHG imaging was used to detect the SBN-lipid membrane patterned substrate [14]. A schematic of the optics setup is shown in Fig. 1. The second harmonic output (532 nm) of a Nd:YAG laser (Continuum, Surelite I, 20 Hz, 7ns) was first directed through a half-wave plate and cube polarizer to adjust the power. The laser beam was then sent through a beam shaper (MoTech GmbH, 6 mm π-shaper) to create a uniform amplitude distribution across the beam profile while maintaining the polarization of the beam. To further homogenize the beam intensity distribution and wave-front a Keplerian telescope was used to bring the beam to a focus. The beam was then resized (beam width of ~3mm) and collimated over a long distance ~2m using a Galileo telescope. After which the beam, with an energy of 20 mJ/pulse as measured at the sample position, was directed on the surface of the prism under total internal reflection. The reflected beam was steered back on itself so as to spatially and temporally overlap with the incident beam. The resulting SHG photons were emitted at
266 nm along the surface normal. A UV solar blind filter (OFIL, Ltd, Israel) was used to allow light only from the SHG signal to be collected.

SHG image acquisition was achieved using a CCD camera (Andor, 1024 x 1024 pixels). Images were taken at 5 different distances from the sample, ranging from 7.6 cm to 40 cm. All SHG images were collected for 60 minutes to produce the final image. The software package Image J (http://rsbweb.nih.gov/ij/index.html) was used to analyze and apply false color to the SHG images. After background subtraction of the minimum pixel intensity from the images, the images were flat-field corrected using the Image J macro available at the Integrated Microscopy Core Facility at the University of Chicago (http://digital.bsd.uchicago.edu/%5Cimagej_macros.html) in order to correct for variations in the illumination beam intensity.

UV back illumination images of the 1951 (USAF) negative resolution test target (chrome pattern on UV transparent glass) were collected using a low pressure mercury UV pen lamp (Beckman Coulter, Inc.) as the light source. The light from the pen lamp was collimated and sent through a fused silica diffuser to create a more uniform intensity profile. A narrow bandpass filter was used to select only the 254 nm line of the mercury lamp. Images were collected using a CCD camera and analyzed as mentioned above.

Total internal reflection fluorescence (TIRF) images where collected using an argon-ion laser (Ion Laser Technology) with a 514 nm output. The PSLBs were imaged by incorporating 1 mol % Rhodamine B-capped-DOPE. TIRF imaging were collected using a modified Olympus microscope [14] with a 4 × objective (Optics for Research). Acquisition of the image was accomplished using a CCD camera (Roper Scientific, 512 x 512 pixels). Images were then taken without the microscope and objective at the same distances as the lens-less SHG images.

4. Results and discussion

SSHG images of an SBN-lipid membrane patterned with the 1951 USAF resolution test target collected without an objective lens measured at 5 distances ranging from 7.6 cm to 40 cm are shown in Fig. 3(A-E). The SH signal generated in these images are from the SBN that has preferentially intercalated into the DOPC bilayer, allowing the pattern to be visualized with negligible signal from the regions void of lipids. For comparison, Fig. 3(F) shows a white light image of the positive USAF test target line-width groups (group 0 and group 1) used to pattern the lipid bilayer. In this experiment elements 3-6 of group 0 were imaged because their line-widths are in the range of spot sizes used in chemical and biological microarrays (280 μm to 397 μm), while the smaller elements of group 1 were imaged to demonstrate the limits of the current lens-less SSHG imaging system before diffraction effects are seen (140 μm to 250 μm). Figure 3(A) shows that all elements of both group 0 and group 1 are discernible at an object to detector distance of 7.6 cm. As the distance between the object and detector increases, the line-widths become less discernible due to increased transversal spreading of the image widths and increased curvature in the wave-front, especially for the smaller line-widths of group 1.
Fig. 3. Lens-less SSHG images of a patterned DOPC bilayer containing SBN using the USAF test target group 0 horizontal lines only (elements 3 through 6) on the left and group 1 horizontal and vertical lines (elements 1 through 6) on the right corresponding to line-widths of $397 \, \mu m$, $355 \, \mu m$, $314 \, \mu m$, $280 \, \mu m$, $250 \, \mu m$, $223 \, \mu m$, $198.5 \, \mu m$, $176.5 \, \mu m$, $157.5 \, \mu m$, and $140.5 \, \mu m$, respectively. Images were recorded at detector-sample distances of (A) 7.6 cm, (B) 15.2 cm, (C) 22.9 cm, (D) 30 cm, and (E) 40 cm. (A white light image of group 0 and group 1 of the USAF test target is shown in (F)).

To confirm that the non-uniformity seen in the lens-less SSHG images shown in Fig. 3 is not a result of the lens-less imaging system, an image of the group 0 elements ($397 \, \mu m$, $355 \, \mu m$, and $314 \, \mu m$) was taken using a convex lens to reconstruct the image (shown in Fig. 4). It is apparent in Fig. 4 that even when a lens is employed the line-widths are non-uniform in nature, which suggests that the non-uniformity seen in the images is not a by-product of the imaging system, but rather an inherent artifact of the objects themselves. Thus, the non-uniformity of the line-widths is most likely due to the non-uniform binding of SBN to the DOPC lipid bilayer and the chemical etching process.

Fig. 4. SSHG images taken using a convex lens of a patterned DOPC bilayer containing SBN using the USAF test target group 0 vertical lines only (elements 3 through 5) corresponding to line-widths of $397 \, \mu m$, $355 \, \mu m$, and $314 \, \mu m$.

Each line from the pattern can be taken as a separate object that generates its own SH signal and is described by its own propagating field. The intensity distribution imaged for each line-width can then be analyzed separately to determine the dependence of the imaged...
width on the propagation distance (Eq. (5)). The image width taken as the full width at half maximum (FWHM) of the peak intensity was determined by fitting intensity profiles for the short axis of the line-widths, where the intensity was averaged along the long axis, to a Gaussian distribution (Eq. (8)) for four representative line-widths (397 µm, 355 µm, 280 µm, and 196 µm).

\[ y = y_c + A \exp \left[ -\frac{(x-b)}{2\sigma'} \right]. \]  

(8)

In Eq. (8), \( y_c \) is the baseline offset from zero, \( A \) is the amplitude at the peak center, \( b \) is the position \((x)\) at maximum intensity or the peak center, and \( \sigma' \) is the variance from the \( x \) value at maximum intensity. The FWHM (2.35\( \sigma \)) was determined for the four elements at each distance. The measured FWHM for each of the four representative elements of the test pattern was plotted for each distance and the results are shown in Fig. 5.

The theoretical imaged beam width for each element was calculated from Eq. (5) for distances up to 50 cm where \( w_0 \) was taken as the line-width from the test target and then compared to the experimentally-determined image widths obtained from analysis of the images in Fig. 3. The experimental and theoretical image widths follow the same general exponential increase with increasing distance; however, the experimentally determined image widths were seen to increase more rapidly with increasing distance. In order to determine how much faster the image widths were spreading with distance, the data points where diffraction was not observed (the 7 data points in green to the left of the dashed line in Fig. 5) were then globally fit to Eq. (9) (resulting fit shown in Fig. 5),

\[ w(z) = w_0 \sqrt{1 + \left( \frac{\lambda \alpha}{\pi w_0^2} \right)^2}, \]  

(9)

where \( w_0 \) was determined by extrapolating the fit to \( z = 0 \), and \( \alpha \) represents the parameter which accounts for the more rapid spreading with distance. The experimentally determined image widths were shown to increase 2.33 times faster than that predicted by Gaussian beam propagation theory. This more rapid increase in image width with increasing distance most likely comes from the imperfect assumption that the incoming light waves are perfectly collimated plane-waves. Although much care was taken to highly collimate the incoming light beams, it is inevitable that some distortion of the wave-front of the incoming beams will exist due to the inherent slightly curved wave-fronts from the incident laser and the disturbances in the beam parameters \( R(z) \) and \( w(z) \) caused by the lenses used to resize and collimate the incoming beams [32]. Despite the more rapid spreading of the image widths seen here, the data fit well to Eq. (9) for each sized line-width at close distances. An interesting observation is then seen for all sized line-widths, a sudden decrease in image width occurs and the data seem to no longer follow the fit to Eq. (9). Since, diffraction would cause a decrease in the expected image width [34], the images were analyzed according to classic diffraction theory to determine whether or not this drop in image width coincided with diffraction effects.
The intensity profiles were analyzed according to classic Fraunhofer diffraction theory [34] in one dimension using Eqs. (10) and (11),

\[ I = I_0 \left( \frac{\sin \beta}{\beta} \right)^2 \]  \hspace{1cm} (10)

\[ \beta = \frac{1}{2} kb \sin \theta. \]  \hspace{1cm} (11)

where \( I \) is the intensity, \( k \) is the wavenumber, \( b \) is the short axis width of the bar and \( \theta \) is the angle of the diffracted ray. Fraunhofer diffraction was used as the object size is much larger than the wavelength of light and the emitted light source is a collimated plane-wave [34]. The Fraunhofer diffraction model was then compared to the Gaussian beam propagation model in examining the image width as a function of distance (results are shown in Fig. 6).
Fig. 6. Gaussian beam propagation fit (blue lines) and Fraunhofer diffraction fit (red lines) to the intensity profile for line-widths of 397 μm, 355 μm, 280 μm, and 196 μm at detector-sample distances of (A) 7.6 cm, (B) 15.2 cm, (C) 22.9 cm, (D) 30 cm and (E) 40 cm. The gray graphs represent where the Fraunhofer diffraction fit best to the data.

Using the concordance test, the distance at which the Fraunhofer diffraction model was found to fit best as compared to the Gaussian beam propagation model coincided with the distance at which the sudden decrease in image width occurred for each sized line-width. The dashed line in Fig. 5 indicates where the Fraunhofer diffraction model was found to fit best compared to the Gaussian beam propagation model. These results are consistent with the predictions that when diffraction occurs the image width is much smaller than expected and begins to change linearly with distance. Theoretically, the distance at which diffraction occurs is the confocal distance. However, even after taking into account the 2.33 times more rapid spreading of the image widths seen here, the calculated confocal distance (Eq. (7)) of roughly 80 cm, 64 cm, 39 cm, and 19 cm for the line-widths of 397 μm, 355 μm, 280 μm, and 196 μm, respectively, is still ~2.5 times longer than where diffraction is visible in our data. This discrepancy can be explained in terms of the degree of curvature of the wave-front. Calculating the radius of curvature (Eq. (6)) and subsequently the degree of curvature at the expected confocal distance the wave-front is found to have a degree of curvature of ~38° for each sized line-width; however, the distance at which diffraction is apparent in our data has the degree of curvature of between ~19° to 20°. This suggests that although theoretically diffraction should not occur until the wave-front has curved 38°, lens-less imaging is much more sensitive to the curvature of the wave-front and diffraction is obvious when there is only a slight deviation from a planar wave-front. Despite this lower threshold for the curvature of the wave-front, the similar limit of degree of curvature (~20°) seen to produce diffraction effects for all sized line-widths is consistent with the predictions of Gaussian beam propagation in which diffraction effects are observed at a much closer distance for smaller
line-widths as compared to larger line-widths. This observation is due to the more rapid transformation of the wave-front from planar to spherical for smaller objects and is evident in Fig. 6.

In order to further verify that the lens-less SHG images shown in Fig. 3 result from the coherent plane-wave nature of the emitted light, lens-less transmission images of the USAF negative pattern (shown in Fig. 7) were collected using an incoherent UV light source to back illuminate sample. As expected these images had visible diffraction even at the closest distance of 7.6 cm for both group 1 and group 0 elements. No discernible image is obtained at distances greater than 22.9 cm for group 0 and group 1, in stark contrast to the SSHG images shown in Fig. 3. Additionally, the background was much brighter due the diffraction and rapid transversal spreading of the beam, making the line-widths difficult to differentiate. Although, this incoherent UV back illuminated control demonstrates diffraction was not seen in the SHG images at close distances a more appropriate control to demonstrate the difference between coherent and incoherent imaging is to use an emissive incoherent light source such as fluorescence imaging to compare to the emissive coherent SHG imaging.

As such, lens-less fluorescence imaging was used to image a 1 mol% Rhodamine labeled USAF patterned DOPC lipid bilayer at the various distances. As opposed to SHG, fluorescence is an incoherent process in which scattering and diffraction of the generated electromagnetic waves distort the wave-front at very short distances such that the amplitude and phase of the beam vary randomly with respect to time and position instantaneously. As a result, the coherence length is significantly shorter, causing considerable divergence of the beam and consequently, distortion of the image, making it impossible to resolve an image without the use of a lens system. A 4x objective lens in combination with a CCD was first used to verify that the CCD was aligned and the pattern was in the detection area; the resulting discernible image is shown in Fig. 8(A). Once the objective was removed, no image was discernible even at the closest distance (7.6 cm) as shown in Fig. 8(B). These results demonstrate that the transversal spreading and radial wave-front caused by the incoherent nature of fluorescence necessitates the use of an objective lens to resolve an image, and therefore confirms that only a collimated, coherent plane-wave source has the ability to image without a lens system. Therefore, even though the experimentally determined confocal
distance of the SSHG lens-less images deviates from the theoretical confocal distance due the slightly curved wave-fronts of the incoming beams, the SH emission is still significantly planar and collimated, giving SSHG the ability to image without a lens system.

Fig. 8. Fluorescence Imaging. (A) Fluorescence image of group 1 elements 3 through 6 of the USAF test target using a 4x objective lens. (B) Lens-less fluorescence image at 7.6 cm object-image shows no visible image.

The image spreading and ultimately diffraction seen at increasing distances for this SSHGI system is not merely classic diffraction, but is a consequence of Gaussian beam propagation, which predicts that with increasing distance the beam will become less collimated, less coherent, and less planar, causing increased transversal spreading or divergence of the beam and diffraction. Despite the more rapid increase in image width and shorter confocal distance observed in the lens-less SSHG imaging due to the non-ideal plane-wave nature of the incoming beams, the image data presented in Fig. 3 and analyzed in Fig. 5 is consistent with the general predictions of optical beam propagation theory for the behavior of a Gaussian plane-wave propagating through space. This not only confirms that surface SHG generates a near-collimated, coherent plane-wave at relatively long distances, but also confirms that lens-less SSHG imaging is possible as a result of the collimated, coherent plane-wave nature of the process which minimizes transversal spreading and diffraction effects. Although, it is important to keep in mind that diffraction is only avoided if the object size is large compared to the wavelength and the object-image distance is shorter than the confocal distance.

5. Conclusion

In summary, we have demonstrated the capabilities of SSHG imaging based on its long coherence length and plane-wave nature to directly image the intensity distribution of a patterned SBN-lipid bilayer without a lens system. The SHG beam propagated through space according to Gaussian beam propagation theory, which was used to analyze the image widths as a function of distance. The distance at which lens-less SSHG imaging was able to resolve images without diffraction effects was shown to be dependent on the object size. Even though diffraction is readily observed at even the closest object-detector distance (7.6 cm) for line-widths of 196 μm, there is no observable diffraction for line-widths of 223 μm at this close distance and line-widths of 397 μm did not show diffraction until ~30 cm. Although the experimentally determined confocal distance deviated from the theoretical confocal distance, the deviation was consistent for all object sizes, indicating that the discrepancy was due to the incoming beams having non-ideal planar wave-fronts. Lens-less SSHG imaging was also seen to be more sensitive to the curvature of the wave-front, producing diffraction effects at a degree of curvature of only 20°. However, the complete inability of fluorescence imaging, an incoherent emissive process, to resolve an image without an objective lens demonstrated that the SH emission was still significantly planar and coherent and that it was this property that permits SSHG lens-less imaging without a lens system. Therefore, the inherent properties (highly collimated, coherent plane-wave) of SSHG allow simple, direct amplitude distribution imaging without a lens system. Although it is true that higher resolution images can be produced when a single lens is used, the noticeable differences between the lens-less coherent SSHG imaging and the lens-less incoherent fluorescence imaging shown here emphasize the
effects the wave-front of the beam have on image formation. The initial object size and
detector-sample distance are also shown to effect imaging. Additionally, being able to image
without a lens reduces optical aberrations, improves the overall photon collection efficiency
and increases the detection area, which can be used to increase throughput. In the field of
biosensors and medical diagnostics where only relative intensities are being used these
advantages could be extremely beneficial, as larger arrays could be directly imaged and
analyzed simultaneously.

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