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Membrane imaging by second-harmonic generation microscopy

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We present a detailed analysis of the generation of second-harmonic radiation from biological membranes labeled with a styryl dye. In particular, we consider the high-numerical-aperture limit appropriate to high-resolution microscopy in which an excitation beam is tightly focused from the side onto a membrane surface. In this limit the active surface area that contributes to second-harmonic generation (SHG) depends only on the tightness of the beam focus and the SHG radiation is confined by phase matching into two well-defined off-axis lobes. We derive expressions for the SHG radiation power, angular distribution, and polarization dependence in the cases of ideal or nonideal molecular alignment in the membrane and uniaxiality of the molecular hyperpolarizability. We define an SHG cross section similar to that used in two-photon-excited fluorescence (TPEF) to permit direct comparison of the two imaging modalities. Finally, we corroborate our results with experiments based on the excitation of a styryl dye in giant unilamellar vesicles with a mode-locked Ti:sapphire laser.

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1. INTRODUCTION
Nonlinear optics is proving to be a powerful tool for biological imaging. Fluorescence microscopy by two-photon excitation has become a laboratory standard, and three-photon excitation has demonstrated its feasibility. More recently, nonlinear microscopies based on radiative harmonic generation have also been applied to the imaging of surfaces and biological samples. Second-harmonic generation (SHG) has been used to image the intrinsic nonlinear susceptibility of muscle tissues and to image living cells labeled with styryl dyes. Third-harmonic generation has also been used to provide microscopic images of cells and plant samples. Harmonic generation is an optical phenomenon involving coherent radiative scattering, whereas fluorescence generation involves incoherent radiative absorption and reemission. As such, fluorescence and harmonic images are derived from fundamentally different contrast mechanisms. Moreover, because of its coherent nature, harmonic radiation is usually highly directional and depends critically on the spatial extent of the emission source, making a full description of harmonic generation more complicated than fluorescence generation. We limit our discussions here to the description of molecular SHG only, with the understanding that many of our techniques may be generalized to higher-order harmonics.

It is well known that SHG of dipolar origin cannot arise from a medium that possesses an inversion symmetry. Therefore molecular SHG is usually studied in geometries in which the radiating molecules are spatially ordered. A common geometry is that of a planar interface or a surface that imparts a preferential orientation to a thin layer of molecules. Typically, the layer is illuminated at an oblique angle by a laser beam, resulting in the emission of SHG beams in well-defined reflection and transmission directions. Because the laser beam is usually unfocused or weakly focused, both the driving laser field and the resultant SHG fields can be treated as simple collimated beams with well-defined wave vectors, and the layer of molecules is most appropriately described in this geometry by a macroscopic surface susceptibility that is independent of the illuminated surface area. In contrast, if SHG is intended to provide microscopic image resolution, the driving laser beam must be focused to a small spot size. The driving field can no longer be considered a simple plane wave as above, and the structure of the resultant SHG radiation becomes critically dependent on the particular geometry of the driving field near the focal center. The use of a macroscopic surface susceptibility to quantify the SHG emission becomes inappropriate in this case, and one must resort to a more refined description of the surface nonlinearity for length...
scales much smaller than the radiation wavelength. In particular, it becomes necessary to redefine a surface non-linearity starting from the level of individual molecular hyperpolarizabilities (or nonlinear molecular cross sections).

In this paper we provide a detailed theoretical description of SHG emission appropriate to a high-resolution scanning microscope configuration that uses a tightly focused excitation beam. The sensitivity of SHG emission to tight focusing has already been recognized for bulk nonlinear crystals. Because our attention is directed to biological imaging we consider here the different geometry of molecular SHG from membrane surfaces, as has been demonstrated experimentally in the imaging of cells and vesicles. We begin by characterizing the SHG emission from a single hyperpolarizable molecule in terms of a nonlinear scattering cross section. We then extend our discussion to the characterization of SHG emission from a planar distribution of molecules. In particular, we adopt a formalism that specifically permits a direct comparison between the radiated powers in the case of SHG and in the corresponding case of two-photon-excited fluorescence (TPEF); the latter is already well established in biological microscopy. Indeed, recent results show that simultaneous SHG and TPEF can be obtained from the same collections of molecules. Finally we corroborate our formalism with experiments based on a full characterization, in terms of power, angular distribution, and polarization, of the SHG radiation from giant unilamellar vesicles labeled with steryl dye. Our research here serves as a follow-up to provide complete theoretical and experimental support for the results presented in Ref. 18.

2. THEORY OF SHG MICROSCOPY

A. Single Molecule

We begin by defining the SHG cross section of a single molecule. The most straightforward definition entails calculating the total radiated SHG power and dividing this by the square of the incident driving field intensity. In particular, a molecule is considered an elemental dipole radiator driven by the excitation light according to its first hyperpolarizability. For simplicity we assume that the excitation field is linearly polarized in the \( \hat{z} \) direction and begin by examining only the \( \beta_{zzz} \) component of this hyperpolarizability, denoted \( \beta \). If the excitation light has frequency \( \omega \), the induced dipole moment at frequency \( 2\omega \) will be given by

\[
\mu_{2\omega} = \frac{1}{2} \beta E_0^2 \hat{z},
\]

where \( E_0 \) is the excitation field amplitude and we have used the Taylor convention. The radiated second-harmonic far field at an inclination \( \psi \) from the \( z \) axis is

\[
E_{2\omega}(\psi) = \frac{\mu_{2\omega} \omega^2}{\pi \varepsilon_0 c^2 r} \sin(\psi) \exp(-2i\omega t) \hat{\psi},
\]

where \( \varepsilon_0 \) is the free-space permittivity, \( c \) is the speed of light, \( r \) is the observation distance from the dipole, and \( t \) is the corresponding retarded time. The resultant power per differential solid angle at an inclination \( \psi \), in units of photons/second, may be expressed as

\[
P_{2\omega}(\psi) = \frac{3}{16\pi} \sigma_{\text{SHG}} \sin^2(\psi) I_\omega^2,
\]

where \( I_\omega \) is the excitation intensity in units of (photons/second)/area and

\[
\sigma_{\text{SHG}} = \frac{4n_2\omega^5}{3\pi n_\infty^2 \varepsilon_0^2 \varepsilon_\infty^2} |\beta|^2
\]

\( (n_\omega \text{ and } n_{2\omega} \text{ are the indices of refraction at } \omega \text{ and } 2\omega; \ h \text{ is Planck’s constant}. \) We have defined \( \sigma_{\text{SHG}} \) such that the total SHG power, obtained by integration of Eq. (3) over all solid angles, reduces to the simple expression

\[
P_{\text{SHG}} = \frac{1}{2} \sigma_{\text{SHG}} I_\omega^2
\]

(the extra factor of \( 1/2 \) stems from our description of powers in units of photons/second rather than in watts).

We recall that the fluorescence power emitted by a dipole undergoing two-photon excitation can be expressed similarly as

\[
P_{\text{TPEF}} = \frac{1}{2} \sigma_{\text{TPEF}} I_\omega^2,
\]

where \( \sigma_{\text{TPEF}} \) is the two-photon fluorescence (or action) cross section, defined by the two-photon absorption cross section multiplied by the fluorescence quantum yield. In view of the similarity between Eqs. (5) and (6), we may regard \( \sigma_{\text{SHG}} \) as the cross section for SHG of an individual dipole. In particular, \( \sigma_{\text{TPEF}} \) and \( \sigma_{\text{SHG}} \) may be expressed in the same units for direct comparison. We note here that \( \sigma_{\text{SHG}} \) depends on the square of the magnitude of the molecular first hyperpolarizability, whereas \( \sigma_{\text{TPEF}} \) depends on the imaginary part of the second hyperpolarizability. As such, \( \sigma_{\text{SHG}} \) tends to be much smaller than \( \sigma_{\text{TPEF}} \) in practice.

B. Coherent Summation

We now turn to the case of a collection of molecules driven by a field \( E_\omega \) and generalize the procedure described above to evaluate the resultant SHG radiation angular distribution and power. We restrict ourselves to the geometry shown in Fig. 1, in which the dipoles are assumed to be uniformly distributed in a two-dimensional \( x-y \) plane (membrane plane) and illuminated by a highly focused excitation beam propagating side-on in the \( \hat{x} \) direction and polarized in the \( y-z \) plane (the fact that we use a side-on geometry rather than a head-on geometry will become evident). Our basic strategy consists in coarse-grain averaging the molecular dipole moments over membrane surface areas whose dimensions are small compared with the radiation wavelength but large enough to encompass large numbers of molecules. In this way we may define a local induced macroscopic dipole moment per unit molecular surface density:

\[
\mu_{2\omega}(x,y) = \frac{1}{2} E_{\omega}^2(x,y) \sum_{j,k} (\beta_{j,k}) \hat{e}_j \hat{e}_k,
\]

where \( E_{\omega}(x,y) \) is the complex amplitude of the driving field at the position \( (x,y) \) on the membrane, \( \hat{e} \) is its polarization direction, \( \langle \beta \rangle \) symbolizes a local ensemble average of the molecular hyperpolarizabilities (we shall describe
Fig. 1. Coordinate system defining the SHG emission direction.

The membrane surface (shaded; $z = 0$) is approximated to be planar at the length scales considered. The focused excitation beam propagates in the $+x$ direction. The resultant SHG is mostly confined to an interaction area schematically depicted as waists, respectively (explicit expressions for these may be found in App. B of Ref. 23, $k_w = n_{2\omega}c$ is the local wave vector, and $\xi$ is a parameter that characterizes the phase shift experienced by a Gaussian beam in the vicinity of a focal center. This phase shift is commonly referred to as a Gouy shift or a phase anomaly. In the case of weak focusing, $\xi$ may be approximated by $(1 - 2k_w^2w_0^2)$, whereas for tight focusing this expression tends to be a slight overestimate (see Appendix A). We emphasize this average in more detail below), and we have neglected local field effects. We emphasize that our term “macroscopic” here is still microscopic relative to wavelength scales. Thus all the molecules taken in the coarse-grain approximation are driven in phase with one another, and the net local dipole moment per unit area generated at this position is simply $N_s\mathbf{\mu}_2\omega(x, y)$, where $N_s$ is the molecular surface density. The phase of $\mathbf{\mu}_2\omega(x, y)$ at different positions is determined by the phase of the driving field $\mathbf{E}_\omega(x, y)$. To calculate the radiation generated by a global membrane surface, we then treat the local dipole moments at each position on the membrane plane as elemental radiators and coherently sum their radiated electric fields while taking their relative phases and amplitudes into account.

As emphasized in Section 1, the excitation field cannot be regarded as a simple collimated beam here, and indeed a full calculation that takes diffraction and polarization into account shows that the field’s amplitude and phase cannot even be expressed analytically in the case of tight focusing. It has been shown, however, that as far as nonlinear interactions are concerned one may closely approximate a tightly focused field by assuming that its amplitude is Gaussian in both the axial and the lateral directions about the focal center and that its phase near the focal center progresses linearly. That is, we can write

$$E_\omega(x, y) = -iE_\omega \exp\left(-\frac{x^2}{w_x^2} - \frac{y^2}{w_y^2} + i\xi k_w x\right),$$

where $w_x$ and $w_y$ are the axial and the lateral field waists, respectively (explicit expressions for these may be found in Ref. 17). The Gouy shift experienced by a Gaussian beam in the vicinity of a focal center. This phase shift is commonly referred to as a Gouy shift or a phase anomaly. In the case of weak focusing, $\xi$ may be approximated by $(1 - 2k_w^2w_0^2)$, whereas for tight focusing this expression tends to be a slight overestimate (see Appendix A). We emphasize

again that the field in Eq. (8) differs from a collimated beam in two respects: It experiences a local axial amplitude variation about the focal center as well as a phase shift. Both of these effects play a considerable role in the structure of the resultant SHG.

By adopting the coordinate system illustrated in Fig. 1, we proceed to calculate the second-harmonic far field radiated in the direction $(\theta, \varphi)$. We begin by writing the contribution to the radiated electric field from the local induced polarization per unit molecular surface density and at the focal center only [indicated by the superscript (0)]; $\mathbf{\mu}_2\omega(0, 0)$]. This is given by

$$\mathbf{E}_{2\omega}^{(0)}(\theta, \varphi) = \mathbf{E}_{2\omega}^{(0)}(\theta, \varphi) = \frac{\eta}{r} \mathbf{M} \cdot \mathbf{\mu}_2\omega,$$

where $\eta = \omega^2 / \pi \varepsilon_0 c^2$ and $\mathbf{M}$ is the projection matrix, defined by

$$\mathbf{M} = \begin{bmatrix} -\sin \theta & \cos \theta \sin \varphi & \cos \theta \cos \varphi \\ \cos \varphi & -\sin \varphi & \cos \varphi \\ -\sin \varphi & \cos \theta \cos \varphi & \cos \theta \sin \varphi \end{bmatrix}.$$  

That is, $\mathbf{E}_{2\omega}^0$ is the amplitude of $\mathbf{E}_{2\omega}$ in the $\hat{\theta}$ direction and $\mathbf{E}_{2\omega}^0$ is the amplitude of $\mathbf{E}_{2\omega}$ in the $\hat{\varphi}$ direction. The total radiated field in the propagation direction $(\theta, \varphi)$ is then given by the coherent summation of the contributions from the local induced polarization at all points $x$ and $y$, with their associated spatially dependent phase shifts taken into account. We find then that

$$\mathbf{E}_{2\omega}(\theta, \varphi) = \frac{\eta N_s}{r} \int \int \mathbf{M} \cdot \mathbf{\mu}_2\omega(x, y) \times \exp\{-i k_{2\omega}(x \cos \theta + y \sin \theta \sin \varphi)\} \, dx \, dy,$$

where $k_{2\omega} = 2n_{2\omega}c$. On integration, Eq. (11) yields

$$\mathbf{E}_{2\omega}(\theta, \varphi) = NA(\theta, \varphi) \mathbf{E}_{2\omega}^0(\theta, \varphi),$$

where we have introduced the parameters

$$N = \frac{\pi}{2} w_x w_y N_s,$$

and $A(\theta, \varphi) = \exp\left(-\frac{k_{2\omega}^2}{8} \left[w_x^2(\cos \theta - \xi')^2 + w_y^2(\sin \theta \sin \varphi)^2\right]\right)$.
center, and the second is an angular modulation term defined by the scalar function $A(\theta, \varphi)$. As we shall see below, the effect of the latter modulation term on the radiation structure is quite dramatic.

Finally, the radiated second-harmonic powers in the $p$ and $s$ polarization and in the solid angle defined by $(\theta, \varphi)$ are given by

$$P_{\text{SHG}}^p = \int_{0}^{2\pi} A_\theta(\theta, \varphi)^2 \varphi^2 \sin \varphi \sin^3 \theta \, d\theta d\varphi,$$

and the total radiated power obtained by integration over all angles becomes

$$P_{\text{SHG}} = \frac{4\pi}{3} n_2 \varepsilon_0^2 \varepsilon_0 c^2 N^2$$

$$\times [\Theta_3 + \Theta_4 + \Theta_5] \frac{1}{2},$$

where we have introduced the angular structure parameters

$$\Theta_3 = \frac{3}{8\pi} \int A^2(\theta, \varphi) \sin^3 \theta \, d\theta d\varphi,$$

$$\Theta_4 = \frac{3}{8\pi} \int A^2(\theta, \varphi) [1 - \sin^2 \theta \sin^2 \varphi] \sin \theta \, d\theta d\varphi,$$

$$\Theta_5 = \frac{3}{8\pi} \int A^2(\theta, \varphi) [1 - \sin^2 \theta \cos^2 \varphi] \sin \theta \, d\theta d\varphi.$$

The contributions to the total power from the various directional components of the induced second-harmonic dipoles are therefore readily identified. We note that if $A(\theta, \varphi) = 1$, as is the case for a single elemental dipole radiator, then $\Theta_3 = \Theta_4 = \Theta_5 = 1$.

### C. Membrane Hyperpolarizability

In the derivation of Eqs. (15) and (16), no assumptions have been made about the dipole vector $p_{2w}(0)$, which may be taken as arbitrary. For our considerations, however, we shall assume that $p_{2w}(0)$ is generated by molecules that are quasi-uniaxial. That is, if we define a coordinate system $(X, Y, Z)$ that is proper to a molecule, then the molecular first hyperpolarizability is dominated largely by the single component $\beta_{zz}$. Moreover, we assume that the molecules are preferentially aligned in the lipid membrane such that, on average, the molecular $Z$ axis is in the same direction as the $z$ axis defined by the membrane, though we do not otherwise specify the distribution that governs tilt angles $\alpha$.

The molecular $X$ and $Y$ axes, in turn, are assumed to be uniformly randomly oriented. In particular, we discount any possible correlation between the $X$ and $Y$ orientations and tilt angle $\alpha$. With the above assumptions in mind, we may derive a coarse-grained local ensemble average for the hyperpolarizability that is valid over regions whose dimensions are small compared with the optical wavelength but large enough to encompass a large number of molecules. Expressed in the membrane coordinate system $(x, y, z)$, the nonzero components of this first hyperpolarizability are given by

$$\beta_{xxx} = \beta_{xzz} = \beta_{yzz} = \beta_{zyz} = \beta_+,$$

$$\beta_{xzy} = \beta_{zyx} = -\beta_{yyz} = -\beta_{yzx} = \beta_-,$$

$$\beta_{zxx} = \beta_{xyz} = \beta_1,$$

$$\beta_{zzz} = \beta_2,$$

where we have used the definitions

$$\beta_+ = \frac{1}{4}(\cos^2 \alpha)B_+ + \frac{1}{2} (\sin^2 \alpha \cos \alpha)(B_Z - B_T),$$

$$\beta_- = \frac{1}{4}(3 \cos^2 \alpha - 1)B_-,$$

$$\beta_1 = \frac{1}{4}(3 \sin^2 \alpha \cos \alpha)(B_Z + B_T - 2B_+),$$

$$\beta_2 = \frac{1}{4}(\cos^2 \alpha)(B_T + 2B_+),$$

where $(\cdot)$ signifies an ensemble average and

$$B_+ = \frac{1}{4}(\beta_{XXZ} + \beta_{YYZ}),$$

$$B_- = \frac{1}{4}(\beta_{XYZ} - \beta_{YZZ}),$$

$$B_T = \frac{1}{4}(\beta_{XXZ} + \beta_{YYZ}),$$

$$B_Z = \beta_{ZZZ}.$$

Having derived the effective molecular hyperpolarizability in the membrane frame, we may now derive the induced second-harmonic dipole at the focal center $p_{2w}(0)$ from Eq. (7). We consider the case in which the driving field is polarized in the $y-z$ plane and write $\hat{E} = (0, \sin \phi, \cos \phi)$, where $\phi$ represents the inclination of the polarization direction from the $z$ axis. A straightforward application of Eqs. (7) and (18) then yields

$$p_{2w}(0) = \frac{1}{2} \epsilon_0 c^2 \frac{\epsilon_0}{\epsilon_0 c^2} \hat{z}.$$

Finally, by inserting Eq. (21) into Eqs. (9), (12), and (15), we obtain the $p$ and $s$ polarization components of the radiated second-harmonic power.

### D. Uniaxial Hyperpolarizability

To distill the basic features of our results, we restrict ourselves here to the simplifying case in which the molecules in the membrane are perfectly oriented in the $z$ direction (i.e., $\alpha = 0$) and strictly uniaxial, such that the only nonzero component to the molecular first hyperpolarizability is $\beta_{ZZZ}$ (i.e., $\beta_+ = \beta_2 = \beta_1 = 0; \beta_2 = \beta_{ZZZ}$). We also begin by considering the case in which the driving field is polarized in the $z$ direction (i.e., $\phi = 0$). This is the same case as examined in subsection 2A for a single molecule, with the only difference being that here we consider the effects of a planar distribution of molecules. As before, we find at the focal center

$$p_{2w}(0) = \frac{1}{2} \epsilon_0 c^2 \frac{\epsilon_0}{\epsilon_0 c^2} \hat{z},$$

which again is the induced dipole moment per unit molecular surface density. The radiated power from the distribution of induced dipole moments about the focal center, expressed in photons/second, is then given by
where the factor \(2/\sqrt{2}\) stems from the volume contrast of a three-dimensionally Gaussian fluorescence excitation volume.23

Because the SHG radiation is emitted into two well-defined lobes, small-angle approximations about \(\theta = \pm \theta_{\text{peak}}\) and \(\varphi = 0\) may be used to provide an estimate of the structure parameter \(\Theta_\theta\). A straightforward evaluation of Eqs. (17) with these approximations yields

\[
\Theta_\theta = \frac{3 \xi^2}{k^2 w_x w_y \sqrt{1 - \xi^2}},
\]

Bearing in mind the approximate dependence of the focal area on the numerical aperture (NA) of the excitation beam \((w_x \sim NA^{-2}; w_y \sim NA^{-1})\), we can infer the following relations: \(N \sim NA^3; I_\text{w} \sim NA^2\), and for low NA we can approximate \(\Theta_\theta \sim NA^2\) (see Appendix A). We find then that the total emitted SHG power is roughly independent of NA for \(NA < 0.8\). For higher NA's, the SHG power diminishes. In comparison, the total emitted TPEF power scales as NA in a two-dimensional geometry.

E. Polarization Anisotropy

In our discussions so far we have assumed that the membrane geometry was planar. In our experiments, however, the membranes are in fact spherical, with diameters typically in the 20–50-μm range. The excitation beam waist at the focal center is typically of submicrometer size; hence the assumption that the membrane is planar over this dimension is entirely valid. We note, however, that the membrane coordinate frame as defined above is not the same as the laboratory coordinate frame. In particular, if the excitation beam polarization is linear and fixed in the laboratory frame, it will appear to tilt in the membrane frame, depending on which portion of the membrane surface is illuminated. If we can scan only along an equatorial cross section of the membrane, the polarization direction will rotate relative to the membrane’s z axis with an angle \(\phi\), which spans 0 to 2π. In the case of perfectly aligned uniaxial molecules \((\beta_+ = \beta_- = \beta_z = 0)\), the induced local dipole moment for an angle \(\phi\) as obtained from Eq. (21) is simply

\[
\rho_\phi = \frac{1}{2} E_\phi^2 (0, 0, \beta_\phi \cos^2 \phi).
\]

The resultant \(p\) and \(s\) polarizations of the radiated field in the local membrane coordinate frame are then obtained from Eq. (9), and the total radiation power generated locally along the membrane equator is found to vary as \(\cos^4 \phi\).

Experimentally it is difficult to isolate the \(p\) and \(s\) components of the radiated field because these are not fixed in the laboratory frame. We can transform them, however, into orthogonal polarization components, which are fixed in the laboratory frame through the relation

\[
\begin{bmatrix}
E_{2w}^p \\
E_{2w}^s
\end{bmatrix} = \begin{bmatrix}
\cos(\varphi - \phi) & -\sin(\varphi - \phi) \\
\sin(\varphi - \phi) & \cos(\varphi - \phi)
\end{bmatrix} \begin{bmatrix}
E_{2w}^p \\
E_{2w}^s
\end{bmatrix}.
\]

Here \(E_{2w}^p\) and \(E_{2w}^s\) represent the radiated electric field components parallel and perpendicular, respectively, to the excitation beam’s polarization direction. These latter polarization components can be isolated quite simply, as is shown below.

Following steps similar to those used in deriving Eq. (16), we can derive the total radiated SHG powers along the \(\parallel\) and \(\perp\) polarizations. These are given by
molecule is excited near resonance. In particular, for an amino)styryl)pyridinium (Di-6-ASPBS). The donor transfer energy Di-6-ASPBS in membrane allows us to infer a charge in membrane are available, the absorption spectrum of the lipophilic styryl dye is made from a pure lipid in water. These serve as model systems for the study of SHG radiation from membrane surfaces. The lipid type is 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti, DOPC), which is in the fluid phase at room temperature. The vesicles are labeled with a dichroic mirror (DM), bandpass (BP) filtered, and detected with a photomultiplier tube (PMT). The laser light is focused into the sample with a water-immersion microscope objective (Olympus, LUMPlanFL60×WIR) and the resultant SHG is collected in the forward direction, while the TPEF is collected in the backward direction. Our sample consists of giant unilamellar vesicles (GUV’s) made from a pure lipid in water. These serve as model systems for the study of SHG radiation from membrane surfaces. The lipid type is 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti, DOPC), which is in the fluid phase at room temperature. The vesicles are labeled with 1 mol. % with Di-6-ASPBS; preparation and labeling are described elsewhere. Calcium ions are added to promote adhesion between adjacent vesicles, and the glass slide is coated with poly-L-lysine (Sigma P8920) to prevent sticking and bursting.

4. EXPERIMENTAL ANALYSIS

A. SHG and TPEF Imaging of Membranes

Our experimental apparatus is a home-built scanning SHG TPEF microscope, the basics of which are shown in Fig. 3. The excitation source is a mode-locked Ti:sapphire laser (Spectra-Physics), which delivers ~80-fs pulses at an 81-MHz repetition rate. The laser light is focused into the sample with a water-immersion microscope objective (Olympus, LUMPlanFL60×WIR) and the resultant SHG is collected in the forward direction, while the TPEF is collected in the backward direction. Our sample consists of giant unilamellar vesicles (GUV’s) made from a pure lipid in water. These serve as model systems for the study of SHG radiation from membrane surfaces. The lipid type is 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti, DOPC), which is in the fluid phase at room temperature. The vesicles are labeled with 1 mol. % with Di-6-ASPBS; preparation and labeling are described elsewhere. Calcium ions are added to promote adhesion between adjacent vesicles, and the glass slide is coated with poly-L-lysine (Sigma P8920) to prevent sticking and bursting.

Figure 4 shows SHG and TPEF images of three GUV’s of nearly equal radii that have adhered to form a foam. The GUV’s are labeled with Di-6-ASPBS and acquired under the conditions described above. SHG scales with the exciting wavelength of 880 nm, we find that β ≈ 1 × 10−47 C m3 V−2, or, equivalently, σSHG ≈ 1 × 10−3 GM (1 GM = 10−50 cm/(phonon s−1)). For comparison, the TPEF cross section of Di-6-ASPBS in membrane at the same excitation wavelength has been estimated to be σTPEF ≈ 30 GM, based on TPEF measurements in ethanol. We note that, despite the fact that σSHG is ~4 orders of magnitude smaller than σTPEF for a single molecule, we may benefit from the fact that SHG field amplitudes add coherently in the case of a large number of molecules, as shown in Eq. (26). In practice and for standard dye labeling densities, we can easily obtain SHG and TPEF powers that are comparable, as we show below.

3. SHG CROSS SECTION

The molecule used in our experimental investigation is the lipophilic styryl dye N-(4-sulfobutyl)-4-(4-dihexylamino)styryl)pyridinium (Di-6-ASPBS). The donor-(π-bridge)-acceptor structure of this molecule permits significant charge transfer along its major Z axis, resulting in a large first hyperpolarizability component βZZZ. Donor-(π-bridge)-acceptor molecules have been found to be well described by a two-state model. Although no direct experimental measurements of βZZZ in membrane are available, the absorption spectrum of Di-6-ASPBS in membrane allows us to infer a charge transfer energy hωeg ≈ 2.56 eV, a transition moment μq ≈ 10 D, and a damping factor Γ ≈ 0.19 eV, all specific to a membrane environment. In addition, previous electrochromism measurements of similar dyes in membrane allow us to estimate that Δμ = 16 D for Di-6-ASPBS. We may therefore predict a large static first hyperpolarizability of roughly β(0) ≈ 2 × 10−46 C m3 V−2. This value is enhanced when the molecule is excited near resonance. In particular, for an

\[ P_{\text{SHG}} = \frac{4\pi}{3} n_{2e} e_0 c \eta^2 N^2 \mu_{2e}^{(0)2} (\Theta_z - 2\Theta_z') \cos^2 \phi + \Theta_z', \]

(30)

\[ P_{\text{SHG}1} = \frac{4\pi}{3} n_{2e} e_0 c \eta^2 N^2 \mu_{2e}^{(0)2} (\Theta_z - 2\Theta_z') \sin^2 \phi + \Theta_z', \]

(31)

where we have introduced the auxiliary angular structure parameter

\[ \Theta_z' = \frac{3}{8\pi} \int A^2(\theta, \varphi) \times (1 - \cos \theta)^2 \sin^2 \varphi \cos^2 \varphi \sin \theta d\theta d\varphi. \] (32)

Although the above relations are exact, in most cases of interest Θz' is considerably smaller than Θz, and can safely be neglected. We then find that the powers emitted into the orthogonal polarization components P_{\text{SHG}1} and P_{\text{SHG}2} vary, respectively, as cos 6 φ and cos 6 φ sin 2 φ along the membrane equator. We emphasize that the above results apply to rigorously uniaxial and well-oriented molecules only. In practice, not all molecules have these attributes, as will be seen below.
square of the excitation intensity and hence leads to the same intrinsic three-dimensional resolution as TPEF, here 510 nm lateral and 1.9 μm axial. An excitation power of less than 1 mW at the sample provides approximately equal measured powers for both SHG and TPEF, allowing the images to be acquired simultaneously with integration times of \(\approx 10\) ms/pixel. The measured radiated powers are in good agreement with those predicted by the model developed in Section 3. In particular, if we estimate our dye surface density to be \(N_s = 1.5 \times 10^{16} \text{ m}^{-2}\), and take into account the fact that essentially all the SHG light is collected because it is highly directional whereas only \(\approx 20\%\) of the radiated fluorescence is epicollected, we find that \(P_{\text{SHG}}/P_{\text{TPEF}} \approx 0.5\). To confirm that the light collected in the forward direction is indeed SHG in origin, we routed both the forward and the backward signals into a spectrograph. The SHG spectrum revealed a sharp peak at 440 nm, whereas the TPEF spectrum was broadly distributed about 580 nm.\(^{18}\) The leftmost two vesicles in Fig. 4(a) have nearly equal TPEF brightness; the third vesicle is less bright, presumably owing to reduced labeling. Because SHG scales with the square of the label surface density, the contrast between the two bright vesicles and the third, dimmer, vesicle is enhanced in Fig. 4(b). We also clearly illustrate here the coherent nature of SHG radiation, as opposed to the incoherent nature of TPEF. In regions where the dipole distributions are symmetric, such as in the adhesion zones between the adjacent vesicles, the SHG vanishes, whereas the TPEF does not. We note that the cancellation of SHG is almost perfect in the left zone. It is less perfect in the right zone owing to the disparity in the surface labeling density.

B. SHG Radiation Pattern

To characterize the angular pattern of the SHG radiation we removed the condenser from our collection optics and replaced it with an oil-immersion microscope objective (Olympus, UPlanFL60×1.25 Oil Iris). We then imaged the back aperture of this objective onto a CCD camera (Cohu 4912/Scion Corporation LG-3 frame grabber). This allowed us to visualize directly the emission angle \(\theta\). Figure 5 illustrates the SHG radiation pattern obtained when the laser scanning was restricted to a small patch of membrane on the equatorial slice of a vesicle. The average power at the focus was maintained below 5 mW, permitting a CCD integration time of \(\approx 5\) ms/pixel. The two lobes that occur at \(\pm \theta_{\text{peak}}\) are manifest. These are the emission directions that correspond to phase matching of the SHG and excitation fields, as predicted by theory. As shown, however, the two lobes are not perfectly cylindrically symmetric about \(\theta = 0\). In particular, the left lobe curves outward rather than inward. This may be the result of the local membrane curvature, which is not taken into account in our theory. In particular, when the membrane curvature was reversed, the lobe symmetry also became reversed. We also point out that there was a slight index-of-refraction mismatch between the insides and the outsides of our vesicles. As a result, the lobe that is directed toward the inside of a vesicle experiences a slight distortion owing to the lensing effect when it exits the vesicle, which may further contribute to the observed asymmetry in the lobes.

When the laser was allowed to scan over an entire vesicle cross section, the angular radiation pattern became annular, as shown in Fig. 6. The radiation pattern was observed to be highly sensitive to the inclination of the local membrane plane relative to the propagation axis. For example, in images generated from cross sec-

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Fig. 4. Simultaneous (a) TPEF and (b) SHG images of three vesicles labeled with Di-6-ASPBS that have adhered to form a foam. The radiating Di-6-ASPBS molecules are more-or-less symmetrically distributed in the adherence regions between the vesicles. In the left region, a symmetric distribution results in a nearly perfect cancellation of SHG. In the right region, the cancellation is imperfect because of a disparity in labeling density. As opposed to SHG radiation, TPEF is independent of molecular distribution because it is incoherent.

Fig. 5. CCD image of the back aperture of the SHG collection objective. The excitation beam is scanned only over a small portion of an equatorial slice of a GUV membrane. The double-peaked angular distribution of the SHG radiation is apparent.
SHG radiation. We perform this selection simply by inserting a linear polarizer between the condenser and the SHG detection photomultiplier tube in Fig. 2. Depending on the orientation of this polarizer relative to the excitation polarization detection, we can effectively isolate the $P_{\text{SHG}}^i$ and $P_{\text{SHG}}^j$ components of the SHG radiation, as described in Subsection 2.E. Our results, for example, for Di-6-ASPBS labeling of GUV’s are presented in Fig. 7. We note that we do not fully recover the respective $\cos^6 \phi$ and $\cos^4 \phi \sin^2 \phi$ angular dependencies for $P_{\text{SHG}}^i$ and $P_{\text{SHG}}^j$, as predicted from our simplified model. Though there may be several explanations for this, the most probable is that the assumptions that Di-6-ASPBS is strictly uniaxial and that it is perfectly aligned in the $\hat{z}$ direction when it is inserted into a membrane are overly simplistic. For example, it has been suggested\(^3^0\) that a molecule closely related to Di-6-ASPBS may possess a significant transverse polarizability component $B_T$ in the molecule frame \(\text{[and hence } \beta_i \text{ in the membrane frame; see Eqs. (18)--(20).}\) Such a transverse component would add a $\sin^2 \phi$ component to $\mu_{2\omega,\omega}$ and hence would significantly modify the expected angular dependencies of $P_{\text{SHG}}^i$ and $P_{\text{SHG}}^j$, yielding a closer fit to experiment. Moreover, even in the case of a rigorously uniaxial polarizability, any deviations from perfect alignment would further complicate matters by introducing a nonzero $\mu_{2\omega,\omega}$ component. Finally, we assumed in Subsection 2.E that the active SHG surface area remained constant everywhere along the membrane equator; that is, we assumed that $w_2 = w_s$ in the focal plane and that the two waist dimensions could be freely interchanged. This is not strictly true in the case of a linearly polarized excitation beam. Suffice it to say that a full polarization analysis of the SHG radiation from membranes remains rather complex owing to the many possible hyperpolarizability components that may be involved, as evidenced by Eqs. (18)--(20). Such a comprehensive analysis is beyond the scope of this paper.

C. SHG Polarization Analysis

To conclude our experimental analysis, we briefly examine some polarization anisotropy properties of the SHG radiation obtained from vesicle membranes. In particular, we consider the case in which the excitation light is linearly polarized and we can select the corresponding parallel and cross-polarized components of the detected SHG radiation.

Fig. 6. Stack of CCD images of the back aperture of the SHG collection objective. The excitation beam, incident from above in the insets, is scanned through full cross sections of a GUV at various latitudes. The equatorial cross section is scanned for image (a), leading to a single annular ring at $\theta_{\text{peak}}$. From image (a) we deduce that $\theta_{\text{peak}} \approx 24^\circ$. The origin of these rings is made clear in the insets, is scanned through full cross sections of a GUV at various latitudes. The equatorial cross section is scanned for image (a), leading to a single annular ring at $\theta_{\text{peak}}$, whereas latitudes below the equator are scanned for images (b) and (c). The CCD images were integrated over times long enough to permit multiple scans.

Fig. 7. Variation of SHG power along the equator of a GUV labeled with Di-6-ASPBS. Excitation is linearly polarized. A polarizer is inserted in the detection path and is oriented parallel ($P_{\text{SHG}}^i$) or perpendicular ($P_{\text{SHG}}^j$) to the excitation polarization. Plots are shown as a function of angle $\phi$ between the normal to the membrane plane ($z$ axis) and the excitation polarization direction. The dashed trace corresponds to the $\cos^6 \phi$ dependence expected for perfectly aligned uniaxial molecules.
5. CONCLUSION

Our purpose in this paper was to lay some theoretical foundations for high-resolution SHG imaging of membranes. Inasmuch as SHG imaging may be performed simultaneously with TPEF imaging, we have taken particular care to establish a formalism that permits direct comparison between the two modalities. Similarities between SHG and TPEF emission powers are that both scale with the square of the excitation intensity, that they may both be defined in terms of commensurate molecular cross sections, and that their active focal areas (or volumes) are independent of the sample size and depend only on the focus tightness. Differences lie in the fact that SHG power scales with the square of the number of molecules, whereas TPEF power scales only linearly with number. Moreover, a structure parameter is necessary for proper quantification of SHG power, which depends on the extent of the active focal area. We note finally that our formalism, which is essentially derived from regarding a distribution of hyperpolarizable molecules as a phased-array antenna, is readily extendible to the generation of harmonics of higher order. In all these cases, effective molecular cross sections may be rigorously defined, and an active focal area (or volume, as in the case of third-harmonic generation in bulk) may be readily identified.

APPENDIX A

A general theoretical framework for describing the electromagnetic field near the focus of a tightly focused beam has been proposed by Richard and Wolf.31 This framework, based on diffraction theory, may be applied to linear or nonlinear optical microscopy. In the case of TPEF microscopy, the signals are incoherent, and only the local field intensity, or correspondingly the field amplitude, need be calculated about the focal center. In the case of SHG microscopy, however, the signals are coherent, and the situation becomes more complicated. Both the local field amplitude and phase must be calculated about the focal center.

It is well known that any focused beam incurs a net $\pi$ phase shift after it passes through its focus. This phenomenon, commonly referred to as a phase anomaly, has a profound influence on the spatial structure of SHG radiation from a membrane. On closer inspection the phase shift, which is $\pi/2$ exactly at the focal center, is found to vary approximately linearly with distance along the propagation axis near the focal center. As such, a focused beam behaves much as a plane wave near the focal center, though with an effective wave vector that has been modified relative to that of an unfocused beam. The effective wave vector of a focused beam near its focal center may be written as $\xi k_w$, where $k_w$ is the wave vector of a corresponding unfocused beam and in general $\xi$ is less than 1. Because the phase of the induced second-harmonic polarizability is governed by the phase of the excitation field, and because appreciable SHG power is produced only in directions that permit proper phase matching between this induced polarizability and the resultant SHG field, we find that, because $\xi < 1$, the radiated SHG power in the forward ($\theta = 0$) direction is weak. The radiated SHG power along $\theta = \pm \theta_{\text{peak}}$, however, is appreciable because the fields in these directions are in phase and add coherently. Both $\theta_{\text{peak}}$ and the total radiated SHG power depend critically on $\xi$; hence a detailed evaluation of this parameter is warranted.

We use the integral formulas derived in Ref. 22 to examine how $\xi$ varies with the NA of the focusing objective of the excitation beam. To begin, we evaluate $\xi$ only at the focal center and consider the case $n_{2w} = n_w$. We may define a focusing angle $\theta_{\text{NA}} = \sin^{-1}(\text{NA}/n_w)$ to characterize the angular range of the excitation light. A comparison of $\theta_{\text{peak}} = \cos^{-1}(\xi)$ and $\theta_{\text{NA}}$ for several NAs is shown in Fig. 8. We observe that $\theta_{\text{peak}}/\theta_{\text{NA}}$ is roughly constant and less than 1, from which we obtain the general rule of thumb that a collection NA equal to the excitation NA is sufficient for essentially all the SHG light to be collected. In particular, for low NAs we find that $\theta_{\text{peak}}/\theta_{\text{NA}} \approx 1$.

![Fig. 8. Variations of the ratio $\theta_{\text{peak}}/\theta_{\text{NA}}$ (where $\theta_{\text{NA}} = \sin^{-1}(\text{NA}/n)$) and of the parameter $(1 - \xi^2)^{1/2}/\xi$ as a function of the NA of a water-immersion microscope objective, assuming a uniformly backfilled aperture and linear polarization. The plots are obtained from full numerical evaluations valid for arbitrary NA. For low NA, $\theta_{\text{peak}} \approx \theta_{\text{NA}}/\sqrt{2}$.](image)

![Fig. 9. Spatial profile of the phase-anomaly parameter $\xi$ (shades) and of the normalized intensity-squared distribution (contours) about the focal center (0, 0) of an 880-nm-wavelength laser beam focused by water-immersion objective with a NA of 0.9. The axial propagation direction is $x$; the lateral direction is $y$. Profiles were calculated assuming a uniformly backfilled aperture and linear polarization. $\xi$ is relatively constant within the major portion of the intensity-squared profile.](image)
\( \theta_{peak} = \frac{\theta_{2\lambda}}{\sqrt{2}} \). We also provide a plot of the parameter
\((1 - \varepsilon^2)^{1/2}/\varepsilon^2 \) as found in expression (27). This parameter
is found to vary linearly with NA for low to modest NAs.

Finally, throughout this paper we have assumed that \( \varepsilon \) is roughly constant over the entire membrane surface area that contributes to SHG radiation. To justify this
assumption we illustrate the numerical evaluation of the \( \xi(x, y) \) near the focal center and compare this with the

corresponding intensity distribution of the excitation
beam (Fig. 9) for our experimental case of interest (NA of
0.9). We observe that \( \xi \) varies only slightly within the
major portion of the intensity-squared distribution, and

hence the approximation that \( \xi \) is constant over the active
surface area in which SHG is produced is quite reasonable.

In our case, we find numerically that \( \xi \approx 0.88 \).

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REFERENCES

s derivation fluorescence microscopy,” Science 248, 73–76

of biochemical fluorophores,” in Topics in Fluorescence
Spectroscopy, J. R. Lakowicz, ed. (Plenum, New York,

3. C. Xu and W. W. Webb, “Multiphoton excitation of mole-
cular fluorophores and nonlinear laser microscopy,” in Topics
in Fluorescence Spectroscopy, J. R. Lakowicz, ed. (Plenum,

4. S. Maiti, J. B. Shear, R. M. Williams, W. R. Zipfel, and
W. W. Webb, “Measuring serotonin distribution in live cells

5. C. Xu, W. Zipfel, J. R. Shear, R. M. Williams, and W. W.
Webb, “Multiphoton fluorescence excitation: new spectral

6. J. I. Dadap, J. Shan, A. S. Weling, J. A. Misewich, A. Na-
hata, and T. F. Heinz, “Measurement of the vector charac-
ter of electric fields by optical second-harmonic generation,”

7. R. Gauderon, P. B. Lukins, and C. J. R. Sheppard, “Three-

8. Y. Gao, P. P. Ho, H. Savage, D. Harris, P. Sacks, S.
Schantz, F. Liu, N. Zhadin, and R. R. Alfano, “Second-
(1997).

optical measurement of membrane potential around single

10. P. J. Campagnola, M. Wei, A. Lewis, and L. M. Loew,

“High-resolution nonlinear optical imaging of live cells by
second harmonic generation,” Biophys. J. 77, 3341–3349
(1999).

11. D. Yelin and Y. Silberberg, “Laser scanning third-
harmonic-generation microscopy in biology,” Opt. Express

12. M. Muller, J. Squier, K. R. Wilson, and G. J. Brakenhoff,
“3D-microscopy of transparent objects using third-

of molecular orientation of monolayer adsorbates by optical
(1983).


York, 1984).


17. A. Lewis, A. Khatchatourians, M. Treinin, Z. Chen, G. Pe-
leg, N. Friedman, O. Bouetvich, Z. Rothman, L. Loew, and
M. Sheres, “Second-harmonic generation of biological inter-
faces: probing the membrane protein bacteriorhodopsin and
imaging membrane potential around GFP molecules at
specific sites in neuronal cells of C. elegans,” Chem. Phys.
245, 133–144 (1999).

18. L. Moreaux, O. Sandre, M. Blanchard-Desce, and J. Mertz,
“Membrane imaging by simultaneous second-harmonic gen-
(2000).

19. A. Willets, J. E. Rice, D. Burland, and D. P. Shelton, “Pro-
blems in the comparison of theoretical and experimental hy-

tation cross section of molecular fluorophores with data from
(1996).

21. N. Bloembergen, Nonlinear Optics, 4th ed. (World Scien-
tific, Singapore, 1965).

22. M. Born and E. Wolf, Principles of Optics, 6th ed. (Perga-

23. J. Mertz, “Molecular photodynamics involved in multi-
photon excitation fluorescence microscopy,” Eur. Phys. J. D

for optimizing the first hyperpolarizability of conjugated or-

25. T. Kogej, D. Beljonne, F. Meyers, J. W. Perry, S. R. Marder,
and J. L. Brédas, “Mechanisms for enhancement of two-
photon absorption in donor–acceptor conjugated chro-

26. D. S. Chemla and J. Zyss, Nonlinear Optical Properties of
Organic Molecules and Crystals (Academic, New York,
1984).

27. J. L. Oudar, “Optical nonlinearities of conjugated mo-
elcules. Stillbene derivatives and highly polar aromatic

28. L. M. Loew and L. L. Simpson, “Charge shift probes of
membrane potential. A probable electrochromic mecha-
nism for ASP probes on a hemispherical lipid bilayer,” Bio-

29. O. Sandre, L. Moreaux, and F. Brochard, “Dynamics of
USA 96, 10,588–10,596 (1999).

of \( \pi \) electrons to second harmonic generation in organic

31. B. Richards and E. Wolf, “Electromagnetic diffraction in op-
tical systems. II. Structure of the image field in aplanatic