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Simultaneous Second-Harmonic Generation And Two-Photon Excited Fluorescence Microscopy

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We demonstrate that simultaneous second-harmonic generation (SHG) and two-photon excited fluorescence (TPEF) can be used to rapidly image membranes labeled with a lipophilic styryl dye. We have developed a model based on the theory of phased-array antennas which shows that the SHG radiation is highly structured and can be roughly of the same power as TPEF. This model provides a definition of a SHG cross-section which can be directly compared to the TPEF cross-section.

Keywords: nonlinear optics; optical second-harmonic generation; SHG; second-harmonic microscopy; two-photon microscopy

Second-harmonic generation (SHG) and two-photon excited fluorescence (TPEF) are nonlinear optical phenomena which scale with excitation intensity squared, and hence give rise to the same intrinsic three-dimensional resolution when used in microscopic imaging. Whereas TPEF microscopy is now widely used in biological imaging [l], SHG microscopy at high resolution has only recently been demonstrated as a tool for imaging of living cells [2,3]. Because SHG is

a coherent phenomenon involving radiative scattering whereas TPEF is an incoherent phenomenon involving radiative absorption and re-emission, the two provide intrinsically different contrasts. We show here the possibility of combining these contrasts in a single scanning microscope. By using a charge-transfer lipophilic styryl dye [4] and exciting near its absorption band, we benefit from a large SHG signal and also significant two-photon absorption, which allow simultaneous and rapid SHG and TPEF imaging of membranes.

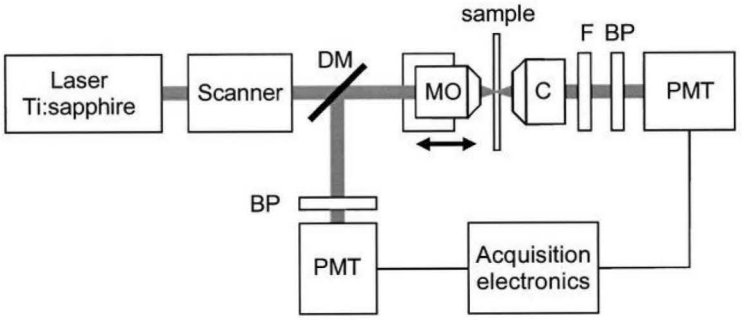

FIGURE 1 SHG and TPEF microscope: a Ti:sapphire laser beam is focussed into a sample with a microscope objective (MO).The transmitted SHG is collected with a condenser (C), bandpass filtered (BP), and detected with a photomultiplier tube (PMT). The transmitted laser light is blocked with a color glass filter (F). The TPEF from the sample is epi-collected, discriminated with a dichroic mirror (DM), bandpass filtered (BP) and detected with a PMT.

Our combined SHG and TPEF microscope is described in Figure 1 and consists of a homebuilt scanning microscope which includes transmitted light detection. The excitation source is a mode-locked Ti:sapphire laser (Spectra Physics) which delivers ~80 fs pulses at a 81 MHz repetition rate. The laser light is focussed into the sample and the resultant SHG is collected in the forward direction while the TPEF is collected in the backward direction. The sample consist of giant

unilamelar vesicles (GUVs) made of a phospholipid in water. The vesicles are labeled at 1-4 mol.% with the lipophilic styryl dye Di-6-ASPBS (N-(4-sulfobutyl)-4-(4-(dihexylamino) styryl)pyridinium); The preparation and labeling of GUV has been described in [5]. The dominating hyperpolarizability component of this molecule is along its charge-transfer axis (molecule-axis), and is denoted β.

When using a tightly focussed excitation beam in TPEF microscopy, the active volume from which fluorescence is generated is sharply confined near the focal center. Similarly, when imaging molecules in a membrane with SHG microscopy, only a small area about the focal center is active. Given the length scales involved, this area may be considered essentially flat and oriented parallel to the excitation propagation direction. The SHG efficacy is highly dependent on the geometry of excitation field near the focal center, and the use of a macroscopic surface susceptibility [6] to quantify SHG emission becomes inappropriate. Therefore, we have developed a model specifically tailored to a tight-focus geometry to characterize SHG in a membrane starting from the level of individual molecular hyperpolarizabilities (or nonlinear cross-sections). Our model is based on the theory of phased-array antennas, in which the dye molecules are regarded as elemental dipole radiators driven at the second-harmonic frequency of the excitation beam, in proportion to their hyperpolarizability. The SHG radiation pattern is derived by taking the excitation polarization to be along the molecular-axis and coherently summing the far-field amplitudes. The molecules are assumed to be perfectly aligned perpendicular to the surface of membrane. The SHG radiation is then found to be double-peaked in well-defined off-axis forward directions which correspond to the angles where the excitation and SHG fields are phase-matched, and critically depend on the phase anomaly of the focussed excitation beam [7]. By integrating the radiation pattern over all solid angles, the total SHG power can be expressed in the simple form:

$$P_{SHG} = \frac{1}{2}\Theta N^2 \sigma_{SHG} \overline{I^2} \tag{1}$$

where N is the effective number of molecules contributing to SHG, $\overline{I^2}$ is the excitation mean square intensity, Θ is a parameter dependent on the focus geometry, and σ_{SHG} is the SHG cross-section for a single molecule. The SHG cross-section is proportional to the molecule's hyperpolarizability squared, and may be expressed as

$$\sigma_{SHG} = \frac{4\hbar\omega^5}{3\pi n \varepsilon_0^3 c^5} |\beta|^2 \quad [\mathrm{m}^4/\mathrm{photon\,s}^{-1}] \tag{2}$$

where n is the index of refraction. We recall that the fluorescence power emitted by N molecules undergoing two-photon excitation with a Gaussian focussed beam can be expressed similarly as

$$P_{TPEF} = \frac{1}{2}\frac{N}{\sqrt{8}}\sigma_{TPEF}\overline{I^2} \tag{3}$$

where σ_{TPEF} is the two-photon fluorescence (or "action") cross-section, defined by the two-photon absorption cross-section multiplied by the fluorescence quantum yield. The above equations lead to a simple expression for the ratio of total SHG to TPEF powers:

$$\frac{P_{SHG}}{P_{TPEF}} \approx \sqrt{8}\Theta\frac{N\sigma_{SHG}}{\sigma_{TPEF}}. \tag{4}$$

The donor-(π-bridge)-acceptor structure of Di-6-ASPBS allows a large charge transfer along the molecular axis [8,9]. Though no direct experimental data on the hyperpolarizability of styryl dyes in membrane is available, based on two-state model [6,10] and experimental measurements [4] we can predict a large near-resonance hyperpolarizability for this molecule when inserted in a membrane and excited at 880 nm, leading to $\sigma_{SHG} \approx 10^{-4}$ GM. In turn, the TPEF cross-section of Di-6-ASPBS in membrane at the same excitation wavelength is estimated to be $\sigma_{TPEF} \approx 30$ GM. Although, σ_{SHG} is small compared to

σ_{TPEF} for a single molecule, the ratio P_{SHG}/P_{TPEF} is significantly enhanced for a large number of molecules owing to the coherent summation of SHG field amplitudes. This ratio is even further enhanced if we consider that SHG power, because of its directional nature, can be more efficiently collected than TPEF power. In our experimental case the number of active molecule under excitation is about $N \approx 2 \times 10^4$, which leads to a power ratio approaching 0.3.

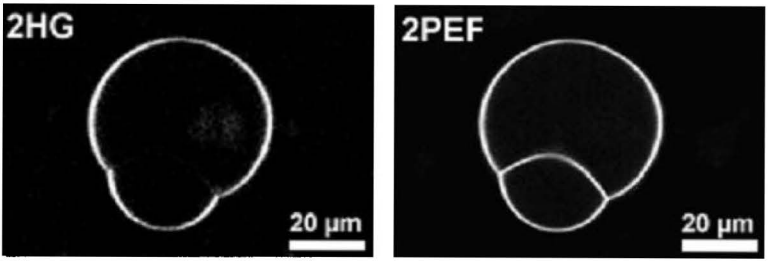

FIGURE 2 TPEF and SHG images of two adhering vesicles labeled with Di-6-ASPBS (equatorial slice), excited at 880 nm. The total acquisition time for the images was 1.5 s, for an excitation power at the sample <1 mW. The adhesion area where the membranes are fused exhibits a centrosymmetric molecular distribution wherein TPEF is allowed but SHG is not.

SHG and TPEF images of Di-6-ASPBS molecules under the conditions described above are illustrated in Figure 2. The large hyperpolarizability of the molecule combined with the coherent summation of SHG resulted approximately equal measured powers in both images, allowing these to be acquired simultaneously. A feature of SHG is that it is a sensitive monitor of local molecular asymmetry. In particular, it is well known that SHG vanishes in the case of symmetric dipole distributions, as illustrated in Figure 2. This sensitivity to local asymmetry is inaccessible to TPEF and promises to be a powerful tool for the study of molecular organization in biological membranes.

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