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Hyper-Rayleigh and Hyper-Raman Scattering Background of Liquid Water in Two-Photon Excited Fluorescence Detection

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The detection of two-photon excited fluorescence is practically free from background caused by linear scattering because two-photon excited fluorescence occurs at a much shorter wavelength region than the excitation light. This property can be used to achieve ultrasensitive fluorescence detection. However, similar to linear scattering in the detection of one-photon excited fluorescence, the question arises whether background caused by nonlinear scattering may limit the detection sensitivity of two-photon excited fluorescence. In this work, quantitative comparisons between two-photon induced scattering of liquid water and two-photon excited dye fluorescence in a standard epifluorescence geometry show that the relative scattering background is typically reduced by orders of magnitude in two-photon excitation as compared to single-photon excitation with confocal detection. Hyper-Rayleigh and hyper-Raman (3400 cm^{-1}) cross sections of liquid water have been measured to be 8×10^{-62} and 7×10^{-63} $cm^4 \cdot s$/photon, respectively, at 840 nm incident wavelength, with absolute values calibrated with respect to the known two-photon fluorescence excitation cross section of fluorescein.

Intrinsic, three-dimensionally resolved, microscopic imaging of dynamical structures and biochemical processes in living preparations has been realized by two-photon laser scanning fluorescence microscopy.[1] In addition to the localized excitation and relatively benign near-infrared illumination wavelengths, detection of two-photon excited (2PE) fluorescence is practically free from background caused by linear scattering because 2PE fluorescence occurs at a much shorter wavelength region than the excitation light. In contrast, the detection sensitivity of one-photon excited (1PE) molecular fluorescence is frequently limited by solvent linear scattering. Although various methods, such as time-gating, have been developed to reduce the scattering background, these methods inevitably compromise detection efficiency of fluorescence signal.

The intense radiation of frequency ν can generate a spectrum of the scattered radiation that includes bands with frequency 2ν and $2\nu \pm \nu_0$, where ν_0 is the frequency associated with a transition between two energy levels of the scattering molecule. The two-photon scattering (2PS) at 2ν is referred to as *hyper-Rayleigh* scattering, that at $2\nu \pm \nu_0$, *hyper-Raman* scattering.[2] Although linear scattering is not a significant component of background in 2PE fluorescence measurements, 2PS background may limit the detection sensitivity of 2PE fluorescence just as linear scattering does in one-photon excitation. Although 2PE fluorescence has been used in a number of applications to achieve high detection sensitivity[3−6] (such as in single-molecule detection), the significance of this nonlinear scattering background in 2PE fluorescence has yet to be evaluated. The first experimental observation of 2PS was made nearly thirty years ago.[7] However, in addition to the difficulties of measuring two-photon cross sections, quantitative measurements of two-photon scattering cross sections are hampered by the inherent weakness of two-photon scattering and competing processes at high intensities. The application of high-repetition-rate femtosecond laser sources, such as the titanium: sapphire (Ti:S) laser, and multichannel detectors now greatly simplifies the measurements of 2PS.[8] Hyper-Rayleigh scattering in solution has been used to determine the hyperpolarizability of nonlinear optical molecules during the last five years.[9] In this paper, we investigate the 2PS background from water solvent in the detection of solute 2PE fluorescence and report cross sections of hyper-Rayleigh and hyper-Raman scattering of liquid water. Our results demonstrate that the relative scattering background of liquid water is typically reduced by orders of magnitude in two-photon fluorescence excitation as compared to single-photon fluorescence excitation with confocal detection.

THEORY

The significance of 2PS background depends on the ratio (r_2) of 2PS cross sections (σ_{2s}) to the 2PE fluorescence cross sections (σ_{2f}), $r_2 = \sigma_{2s}/\sigma_{2f}$. We have measured σ_{2f} for many common fluorophores in the Ti:S tuning range.[10] We present measurements of hyper-Rayleigh and hyper-Raman scattering cross sections of liquid water at 840 nm. In particular, we compare quantitatively the ratios of scattering to fluorescence in both one- (r_1) and two-photon (r_2) excitation.

In the absence of ground state depletion and photobleaching, the amount of 2PE fluorescence (F_2) detected from a single excitation pulse takes the form[10]

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$$F_2 = \frac{1}{2}\sigma_{2f} C_f \eta \phi_f \int_{\text{sample}} dV \int_{-\infty}^{\infty} I^2(t,\mathbf{x})\, dt \qquad (1)$$

where C_f is the fluorophore concentration, η is the fluorescence quantum efficiency, ϕ_f is the overall fluorescence detection efficiency, and $I(t,\mathbf{x})$ is the incident photon flux density as a function of time and space. The factor $^1/_2$ reflects the fact that two photons must be absorbed for each transition event. A similar expression can also be obtained for the 2PS signal (S_2). Replacing the 2PE fluorescence cross section (σ_{2f}) with the 2PS cross section (σ_{2s}) in eq 1, we have:

$$S_2 = \frac{1}{2}\sigma_{2s} C_s \phi_s \int_{\text{sample}} dV \int_{-\infty}^{\infty} I^2(t,\mathbf{x})\, dt \qquad (2)$$

where C_s is the concentration of the scattering molecule and ϕ_s is the overall detection efficiency for the scattering signal. The ratio of 2PS cross sections and the 2PE fluorescence cross sections can be obtained from eqs 1 and 2:

$$r_2 = \frac{\sigma_{2s}}{\sigma_{2f}} = \frac{S_2}{F_2}\frac{C_f \eta \phi_f}{C_s \phi_s} \qquad (3)$$

Thus, by experimentally measuring the ratio of 2PS and 2PE fluorescence in the same experimental setup one can obtain not only σ_{2s}/σ_{2f} but also the absolute values of σ_{2s}, provided that σ_{2f} is already known. By parallel analysis, the ratio of one-photon scattering cross sections to the one-photon fluorescence cross sections is readily shown to be

$$r_1 = \frac{\sigma_{1s}}{\sigma_{1f}} = \frac{S_1}{F_1}\frac{C_f \eta \phi_f}{C_s \phi_s} \qquad (4)$$

EXPERIMENTAL METHODS

Experiments were performed with a mode-locked femtosecond Ti:S laser (SpectraPhysics) in a standard epifluorescence geometry (Figure 1). The output pulse width of the laser was ~100 fs with a repetition rate of 80 MHz. The beam was ~2 mm in diameter ($1/e^2$) at the back of a high numerical aperture (NA) objective (Zeiss Neofluar 1.2 NA 40×, water immersion). Linear polarized light was used throughout the experiments. A long-pass dichroic mirror (DC) with reflectivity of >95% for λ < 610 nm separated the signal (2PS or 2PE fluorescence) from the excitation light. To ensure that the total generated signal did not depend on the sample thickness, we used samples that were significantly thicker (0.5 mm) than the Rayleigh length of the Gaussian beam (~6 μm).[10] With the 1.2 NA objective, the detection solid angle includes ~30% (~1.14π) of the entire space (4π). Signal was focused into an optical fiber connected to an imaging spectrograph. A spectral resolution of 1 nm and a range of 600 nm were achieved with a 150 line/mm grating. Spectra were detected by a liquid nitrogen cooled CCD. An average power of 180 mW at the sample was used to obtain 2PS spectra, corresponding to a peak intensity at the focal point of ~0.6 TW/cm^2. The 2PS signal of liquid water (filtered and deionized) excited at 840 nm was compared to the 2PE fluorescence of fluorescein in water (15 μM, pH ~13). One-photon experiments were carried out in the same experimental setup with an air-cooled argon laser as the excitation source. The one-photon scattering signal of the same water sample was compared to the 1PE fluorescence of rhodamine B (290 nM, water). The wavelength dependence of the transmission of the filter, the fiber, and the spectrograph and the quantum efficiency of the CCD were calibrated using a color-temperature lamp. (We chose rhodamine B instead of fluorescein in one-photon experiments so that the fluorescence and the 3400 cm^{-1} Raman band of liquid water occur at approximately the same wavelength, making our results less sensitive to the calibration of the instruments as mentioned above.)

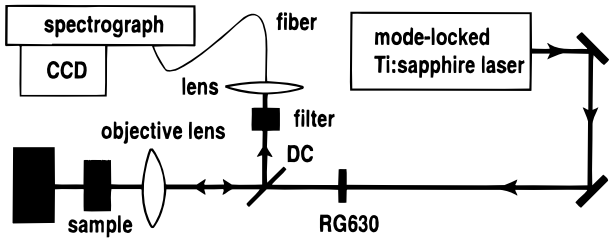

Figure 1. Schematic drawings of the experimental setup. RG630, red pass filter (>630 nm); DC, dichroic mirror.

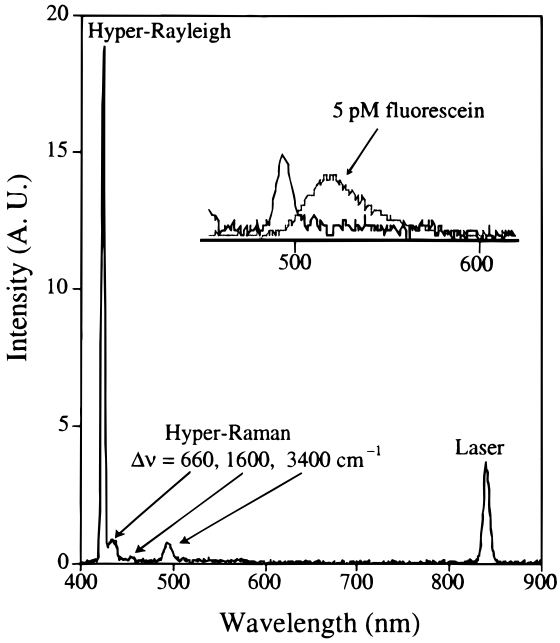

Figure 2. Hyper-Rayleigh and hyper-Raman spectrum of liquid water obtained with an integration time of 120 s. The inset shows the comparison between 5 pM fluorescein emission spectrum and the 3400 cm^{-1} hyper-Raman band.

RESULTS AND DISCUSSION

Figure 2 shows the 2PS spectrum of liquid water with excitation at 840 nm. A blank control was performed under identical experimental conditions but without water in the sample chamber. The resulting spectrum was featureless, and the total number of detected photons was negligible. For quantitative comparison, the inset of Figure 2 shows the 2PE fluorescence emission spectrum of 5 pM fluorescein, extrapolated from the 15 μM fluorescein solution and scaled to the same integration time and excitation power. Integrating the areas of the corresponding signal peaks, the ratios of 2PS cross sections of liquid water to the fluorescein two-photon fluorescence cross section can be obtained (eq 3). Assuming an isotropic distribution of the scattering intensity and using $\eta = 0.9$,[11] we find $r_2 = 6.4 \times 10^{-13}$ for the hyper-Rayleigh band and $r_2 = 5.6 \times 10^{-14}$ for the 3400

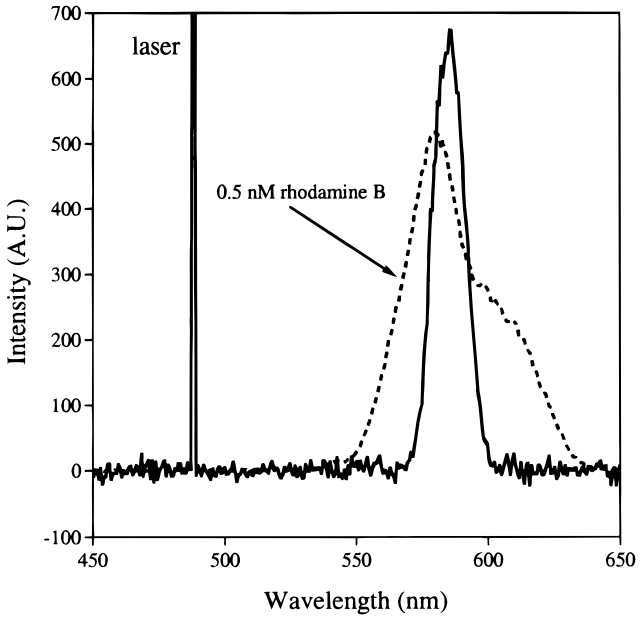

Figure 3. Comparison between the 3400 cm^{-1} Raman band (solid line) and the emission of 0.5 nM rhodamine B (dashed line). The Raman spectrum is obtained with ~2.5 mW excitation power at the sample and an integration time of 2 s. The 1PE emission spectrum of the 0.5 nM rhodamine B is extrapolated from the 290 nM rhodamine B solution and scaled to the same excitation power.

cm^{-1} hyper-Raman band. The absolute two-photon excitation cross section of fluorescein at 840 nm has been previously measured to be $(1.2 \pm 0.3) \times 10^{-49}$ $cm^4 \cdot s/photon$.[10] Thus, the absolute hyper-Rayleigh and hyper-Raman cross sections (3400 cm^{-1} band) of liquid water are estimated to be $(8 \pm 2) \times 10^{-62}$ and $(7 \pm 2) \times 10^{-63}$ $cm^4 \cdot s/photon$, respectively, again assuming an isotropic distribution of the scattering intensity.

An issue of important practical interest is the relative values of r_1 and r_2. Figure 3 shows the 3400 cm^{-1} Raman band with one-photon excitation at 488 nm (solid line). For comparison, the emission spectrum of rhodamine B is also shown. Using eq 4 and $\eta = 0.35$,[12] we obtain $r_1 = 4.1 \times 10^{-12}$. Using the known one-photon cross section of rhodamine B, the Raman cross section of liquid water (3400 cm^{-1} band) can be estimated to be 2.5×10^{-28} cm^2, again assuming an isotropic scattering distribution. Using the known one-photon cross section of fluorescein, this result corresponds to $r_1 = 1.2 \times 10^{-12}$ for fluorescein. Thus, our measurements demonstrate that the hyper-Raman scattering background by water solvent relative to two-photon excitation of fluorescein is reduced by ~20-fold as compared to the Raman scattering of water in one-photon excitation.

Although fluorescein is used as an example in comparing the scattering background from water solvent in one- and two-photon excitation, the relative reduction in scattering background using two-photon excitation should be observed for a variety of fluorophores. Our measurements of more than 25 two-photon excitation spectra of a wide range of molecular fluorophores showed that σ_{2f}/σ_{1f} are $\sim 10^{-32}$–10^{-34} $cm^2 \cdot s/photon$.[10,13] Thus, our measured hyper-Raman to Raman cross section ratio ($\sigma_{2s}/\sigma_{1s} \sim 3 \times 10^{-35}$ $cm^2 \cdot s/photon$) indicated that scattering background from the strongest Raman band of liquid water is typically reduced by 10–100-fold in two-photon excitation as compared to one-photon excitation. No attempt was made to gauge the (one-photon) Rayleigh scattering cross section in this work. In general, Rayleigh scattering cross sections are $\sim 10^3$ times larger than the (one-photon) Raman scattering cross section.[14] Consequently, the background caused by hyper-Rayleigh scattering in two-photon excitation is expected to be 3–4 orders of magnitude lower than Rayleigh scattering in one-photon excitation. We note that the blue-shifted two-photon excitation peak of many fluorophores[10,13] provide the opportunity to further reduce the background; the relatively large spectral separation between scattering and fluorescence allows scattering to be readily distinguished using long-pass filters.

It should be noted that, unlike the isotropic fluorescence emission for small dye molecules (such as fluorescein) in solution, the angular distributions of scattering depend on the incident polarization and symmetry of the scattering molecules and are in general not isotropic. Therefore, the ratios between scattering and fluorescence and the estimated Raman, hyper-Raman, and hyper-Rayleigh scattering cross sections may vary in different detection geometries. However, the relative signals of the hyper-Rayleigh and the three characteristic Raman bands of water observed in our experiment are similar to those observed in a 90° detection setup.[7] Moreover, the epifluorescence geometry used in our experiments is the most common configuration in high-sensitivity fluorescence detection and imaging. Thus, our quantitative comparisons of fluorescence and scattering are of practical importance.

It is well-known that molecular scattering cross sections scale inversely as the fourth power of the wavelength of the scattered light (not the wavelength of the incident light).[2] Thus, σ_{2s}/σ_{1s} would have been smaller by a factor of 1.8 if the incident wavelength for the one-photon experiments was 410 nm instead of 488 nm.

In summary, we have compared scattering background by liquid water in 1PE and 2PE fluorescence detection. Using our measured fluorescein two-photon excitation cross section as a standard, we estimate the hyper-Raman and hyper-Rayleigh cross sections of liquid water at 840 nm. Our results demonstrate, experimentally for the first time, that scattering background by liquid water is typically reduced by orders of magnitude in two-photon excitation as compared to one-photon excitation.

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