Comparison of penetration depth between two-photon excitation and single-photon excitation in imaging through turbid tissue media

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We show, both theoretically and experimentally, that for a turbid tissue medium where Mie scattering is dominant, multiple scattering not only reduces the illumination power in the forward direction but also exhibits an anisotropic distribution of scattered photons. Thus, a signal level under two-photon excitation drops much faster than that under single-photon excitation although image resolution is much higher in the former case. As a result, the penetration depth under two-photon excitation is limited by the strength of two-photon fluorescence and is not necessarily larger than that under single-photon excitation. © 2000 American Institute of Physics.

Two-photon (2p) scanning fluorescence microscopy has been widely used in various fields because this technology has proven advantageous over single-photon (1p) scanning fluorescence microscopy. First, 2p excitation is a nonlinear process and therefore the fluorescence intensity is directly proportional to the square of the excitation intensity. Due to this quadratic dependence, the 2p imaging technique provides a pinpoint excitation/detection method at a deep position within thick samples. Second, if an infrared laser beam is employed for 2p excitation, 2p fluorescence microscopy offers access to ultraviolet (UV) excitation without using UV lasers, and reduces Rayleigh scattering appreciably provided that the size of scatterers in tissue is much smaller than the illumination wavelength. According to these properties of 2p excitation, it has been claimed that 2p excitation results in a deeper penetration depth than 1p excitation.

Recently, 2p fluorescence microscopy has been used in imaging through thick tissue for photodynamic therapy and early detection of small tumors. Biological tissue is usually composed of small scatterers such as bacteria, viruses, malignant cells and so on. The size of these scatterers varies from 0.1 μm to a few micrometers. Therefore, the dominant scattering effect caused by these scatterers is Mie scattering rather than Rayleigh scattering. The physical difference between these two types of scattering is that the former is anisotropic scattering whereas the latter is isotropic scattering. The strength of Rayleigh scattering is inversely proportional to the fourth power of the illumination wavelength. However, Mie scattering exhibits a more complicated nature as shown in Fig. 1.

Figure 1 shows the scattering efficiency Q, which is defined as the ratio of the scattering cross section \( \sigma_s \) to the geometrical cross section, and the anisotropy value g in Mie scattering as a function of the scattering parameter A (A is defined as the ratio of the size of a scattering particle \( a \) to the light wavelength \( \lambda \)). It is seen from this example that, for a given scatterer size, Q and g decrease with the illumination wavelength if A is approximately less than one. The decrease in the scattering efficiency implies a reduction of multiple scattering events, which leads to high image resolution and signal level in microscopy imaging. However the decrease in the anisotropy value results in a broad distribution of scattered photons in the focal region, which may reduce the image resolution and signal level. The competition of these two processes, together with the quadratic dependence of the 2p fluorescence intensity, determines the limiting factor on penetration depth.

To demonstrate the limiting factor on the penetration depth under 2p and 1p excitation, we consider a turbid medium consisting of scattering particles (diameters of 0.202 μm) suspended in water. The turbid medium was placed in a glass cell with lateral dimensions of 2 cm×1 cm. The thickness, \( d \), of the glass cell was varied from 25 up to 250 μm which is the maximum working distance of the objective used in experiments.

A uniform fluorescent polymer bar was embedded at the bottom of the glass cell. The fluorescent bar can be excited under 1p (\( \lambda_s = 488 \text{ nm} \)) and 2p (\( \lambda_s = 800 \text{ nm} \)) excitation with a peak fluorescence wavelength approximately at 520 nm. Due to the different wavelengths associated with 1p excitation, 2p excitation and fluorescence, the

\[ Q = \frac{\sigma_s}{\pi a^2}, \quad g = \frac{\sigma_{\text{g}}}{\sigma_{\text{g}}}, \]

where Q is the scattering efficiency and \( \sigma_{\text{g}} \) is the geometric cross section. The anisotropy value g is defined as the ratio of the size of the scattering particle \( a \) to the light wavelength \( \lambda \), and depends on the refractive indices of the scatterers and the immersion medium.

![FIG. 1. Scattering efficiency Q and anisotropy value g in Mie scattering as a function of the scattering parameter A. The refractive indices of scatterers (spherical particles) and the immersion medium (water) are 1.59 and 1.33, respectively.](image)

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scattering cross section $\sigma_s$, the scattering mean-free-path (SMFP) length $l_s$, and the anisotropy value $g$ are different and can be calculated using Mie scattering theory\textsuperscript{10} (Table I).

The prepared sample cell was placed under an Olympus confocal scanning microscope (Flouview).\textsuperscript{5} For 1p excitation, an Ar ion laser at a wavelength of 488 nm was used. A Spectra-Physics ultrashort pulsed laser (Tsunami) with a pulse width of 80 fs was employed for 2p excitation at 800 nm wavelength.\textsuperscript{5} To avoid the effect of refractive-index mismatching between the turbid medium and the cover glass of the cell, a water-immersion objective (Olympus UplanApo 60×, $\approx$1.13–0.21, numerical aperture=1.2, working distance=250 μm) was used. In the case of 1p excitation, a pinhole 300 μm (optical unit $\sim$3) in diameter was placed in front of the detector to produce an optical sectioning effect with strength similar to that under 2p excitation without using a pinhole.\textsuperscript{1,13} This arrangement implies that the ability of rejecting scattered photons caused by the optical sectioning effect is the same in the two cases.\textsuperscript{14}

Table I

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Relative particle size, $a/\lambda$</th>
<th>Scattering efficiency, $Q_s = \sigma_s/\sigma_s$</th>
<th>Scattering cross section, $\sigma_s$ ($\mu m^2$)</th>
<th>SMFP length, $l_s$ ($\mu m$)</th>
<th>Anisotropy value, $g$</th>
</tr>
</thead>
<tbody>
<tr>
<td>488</td>
<td>0.2069</td>
<td>0.1586</td>
<td>$5.07 \times 10^{-3}$</td>
<td>35.7</td>
<td>0.54</td>
</tr>
<tr>
<td>520</td>
<td>0.1942</td>
<td>0.1356</td>
<td>$4.34 \times 10^{-3}$</td>
<td>41.8</td>
<td>0.482</td>
</tr>
<tr>
<td>800</td>
<td>0.1263</td>
<td>0.0389</td>
<td>$1.24 \times 10^{-3}$</td>
<td>145.38</td>
<td>0.20</td>
</tr>
</tbody>
</table>

To understand the phenomenon in Fig. 2, we should point out that both unscattered and scattered illumination photons can contribute to fluorescence emission. However, the fluorescence contributed by these two groups of photons behaves in different ways between 2p and 1p excitation.

We first consider the case where the thickness $d$ is less than the 1p SMFP length. In this case, the number of unscattered illumination photons is considerable in the total number of photons but decreases exponentially with $d/l_s$. In other words, the log of the emitted fluorescence signal contributed by unscattered illumination photons decreases linearly with $d$, as observed in Fig. 2. Due to the quadratic dependence of 2p fluorescence on the illumination intensity, the number of 2p fluorescence photons produced by the unscattered illumination photons decreases exponentially with $2d/l_s$. As a result, for a given depth $d$, the 2p fluorescence signal excited by the unscattered illumination photons is stronger, because the 2p SMFP length is approximately four times as large as that for 1p excitation (Table I).

When $d$ becomes larger than the 1p SMFP length, the 2p fluorescence signal still decreases exponentially with $2d/l_s$, but scattered illumination photons make a significant contribution to the generation of 1p fluorescence. As a result, the dropping of 1p fluorescence becomes slower than that of 2p fluorescence.

Once the thickness $d$ is larger than the 2p SMFP length, scattered photons play a dominant role in the excitation and emission processes. In general, scattered photons distribute in a broad region near the geometric focus.\textsuperscript{11} Because of the lower anisotropy value $g$ and the larger $l_s$ value, the scattered illumination photons under 2p excitation distribute in a broader region than those under 1p excitation. This feature results in a lower photon density near the geometric focus. Therefore, the 2p fluorescence emission excited by scattered photons is less efficient than the 1p fluorescence emission. Further, due to the quadratic dependence under 2p excitation, 2p fluorescence emission excited by scattered illumination photons becomes even less efficient. In addition, 2p fluorescence photons excited by scattered illumination photons have less of a possibility of reaching the detector because they originate from a broader region. As a result, 2p fluorescence

![FIG. 2. Signal level under 2p and 1p excitation as a function of the penetration depth in a turbid medium consisting of scatterers 0.202 μm in diameter.](image-url)
produced by the scattered illumination photons exhibits a lower signal level than 1p fluorescence.

However the stronger suppression of the contributions from scattered illumination and fluorescence photons under 2p excitation may be advantageous in terms of image resolution. To confirm this, we measured the transverse edge resolution. To confirm this, we measured the transverse edge resolution. To confirm this, we measured the transverse edge resolution. To confirm this, we measured the transverse edge resolution.

The dependence of resolution points, measured from the edge responses after they are fitted, is defined as the distance between the 10% and 90% intensity points, measured from the edge responses after they are fitted. The dependence of resolution on sample thickness is depicted in Fig. 3 which also includes the Monte Carlo simulation results corresponding to the experimental condition. A good agreement between the experiments and theoretical predictions is observed. For the turbid medium we used, the image resolution under 2p excitation is two orders of magnitude higher than that under 1p excitation.

Experiments similar to Figs. 2 and 3 were also carried out for different sizes of scatterers. In general, the smaller the scatterer size (the smaller the anisotropy value and the larger the SMFP length), the quicker the reduction of the 2p fluorescence signal. Based on this property, we can conclude that, for a real tissue medium consisting of different size Mie scatterers, the 2p fluorescence signal level is lower than the 1p signal level at a deep depth. This conclusion is demonstrated in Figs. 4 and 5.

Figures 4 and 5 show the transverse (x-y) and axial (x-z) autofluorescence images of muscle tissue under 2p ($\lambda_s=800$ nm) and 1p ($\lambda_s=488$ nm) excitation. The image intensity has been normalized by the maximum intensity at the surface of the sample. The muscle tissue has an average 1p SMFP length of approximately 20 $\mu$m. The 2p transverse images [Figs. 4(a)–4(d)] were recorded at depths of 10, 30, 50, and 70 $\mu$m, and show a higher resolution but become significantly weaker at a deeper depth than the 1p images [Figs. 4(e)–4(h)]. It can be seen from the axial images (Fig. 5) that, although the degradation of 2p resolution is not pronounced, the 2p fluorescence signal [Fig. 5(a)] decreases faster than the 1p fluorescence signal [Fig. 5(b)].

In conclusion, the penetration depth of 2p excitation in imaging through a tissue medium is smaller than that of 1p excitation because of the lower signal level in the former. However, within the depth of detectable signal, 2p excitation leads to image resolution approximately two orders of magnitude higher than that under 1p excitation.

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3 S. Hell, Bioimaging 4, 121 (1996).
10 C. F. Bohern and D. R. Huffman, Absorption and Scattering of Light by Small Particles (J. Wiley, New York, 1983).