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Limiting Factors on Image Quality in Imaging through Turbid Media under Single-photon and Two-photon Excitation

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Abstract: The effect of multiple scattering in a turbid medium on single-photon and two-photon fluorescence microscopy is experimentally investigated for different scattering characteristics including scattering anisotrophy and optical thickness of a turbid medium. It is demonstrated that two-photon excitation can provide significant improvement in penetration depth through turbid media, due to reduced scattering experienced by the excitation beam. It is also shown that the limiting factor in obtaining high-quality images under single-photon excitation is the fast degradation of image resolution caused by multiple scattering, while for two-photon excitation it is limited by the degradation of image contrast due to the reduction in fluorescence strength.

Key words: turbid media, two-photon excitation, single-photon excitation, fluorescence microscopy, confocal microscopy; penetration depth

Introduction

Two-photon (2-p) fluorescence microscopy has been widely used due to its significant advantages (e.g., reduced scattering, ultraviolet excitation, inherent optical sectioning, reduced photodamage, and phototoxicity) over single-photon (1-p) fluorescence microscopy (Denk et al., 1990). Most fluorescence microscopy work has been performed on thin slices (~10–20 μm thick), in which case, optical multiple scattering is minimal. However, in the case of in vivo imaging (Masters et al., 1997; Schilders and Gu, 1999), the required information may be at significant depths within a biological specimen and thus multiple scattering can have a critical effect on image quality under 2-p excitation.

Recently, the effect of multiple scattering in a turbid medium on image quality under 1-p and 2-p excitation has been investigated (Blanca and Saloma, 1998; Daria et al., 1998). In particular, attention has been paid to the reduction of illumination power due to multiple scattering. In fact, another important issue is how multiple scattering affects image resolution and contrast under 1-p and 2-p excitation. In this article, we report on a detailed experimental investigation into the effect of multiple scattering in a turbid media on image quality under 1-p and 2-p excitation. Image quality is characterized by the sharpness of images of

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Table 1. Calculated Values of the Scattering-mean-free-path Length l_s and the Anisotropy Value g for Four Types of Polystyrene Beads[a]

	Single-photon excitation, λ = 488 nm			Two-photon excitation, λ = 800 nm			Fluorescence, λ = 520 nm		
Sphere diameter, κ (μm)	Scattering cross-section, σ_{s2} (μm^2)	smfp length, l_{s1}	Anisotropy value, g_1	Scattering cross-section, σ_{s2} (μm^2)	smfp length, l_{s2} (μm)	Anisotropy value, g_2	Scattering cross-section, σ_{sf} (μm^2)	smfp length, l_{sf} (μm)	Anisotropy value, g_f
0.107	2.16×10^{-4}	124.9	0.146	6.02×10^{-3}	786.3	0.054	1.78×10^{-4}	151.3	0.131
0.202	5.07×10^{-3}	35.7	0.54	1.24×10^{-3}	145.38	0.20	4.34×10^{-3}	41.8	0.482
0.48	2.15×10^{-1}	11.3	0.86	7.59×10^{-2}	32.05	0.73	1.91×10^{-1}	12.7	0.851
1.056	3.06	8.6	0.93	1.75	15.16	0.90	2.95	8.9	0.92

smfp: scattering-mean-free-path.

[a]Values at wavelengths λ of 488 nm, 800 nm, and 520 nm for a given particle weight concentration of 2.5%.

a bar embedded in turbid media consisting of different types of scattering particles.

Materials and Methods

Experimental work was carried out on an Olympus (Tokyo, Japan) confocal scanning microscope, Flouview (Schilders and Gu, 1999). For 1-p excitation, an Ar ion laser at wavelength 488 nm was used. A Spectra-Physics ultrashort pulsed laser, Tsunami, was employed for 2-p excitation at wavelength 800 nm (Schilders and Gu, 1999). The numerical aperture of the imaging objective (Zeiss FLUAR 20×, ∞/0.17) used for all imaging was 0.75. The incident power for both 1-p and 2-p excitation was approximately 3 mW at the entrance aperture of the imaging objective. In the case of 1-p excitation, a pinhole of 300 μm in diameter was placed in front of the detector to produce an optical sectioning effect similar to that under 2-p excitation without using a pinhole (Gu, 1996).

A 1-mm wide fluorescent polymer bar was embedded within a turbid medium. The peak fluorescence wavelength of the bar was measured to be approximately 520 nm under 1-p and 2-p excitation. Image resolution α is defined as the distance between the 10 and 90% intensity points, measured from the edge responses after they are fitted.

Four types of polystyrene beads suspended in water were used as turbid media. The diameter κ of the four types of beads is shown in Table 1. The range of the bead size chosen was consistent with that of the scattering particle size in real biological samples which do not include large scattering particles (>1 μm) (Morgan et al., 1997). Due to different wavelengths associated with 1-p excitation, 2-p excitation, and the fluorescence, the scattering-mean-free-path (SMFP) length l_S and the anisotropy value g are different. They were calculated using Mie scattering theory (Bohern and Huffman, 1983) for the four sizes of polystyrene beads (see Table 1). Each type of polystyrene bead was placed in a glass cell with lateral dimensions of 2 cm × 1 cm. The glass cell thickness d was varied from 25 μm up to 300 μm.

Results and Discussion

When a fluorescent bar is embedded in a cell consisting of distilled water, images in this case are constructed purely by unscattered photons obeying the prediction by diffraction theory and therefore show the maximum resolution achievable with the imaging system used (Gu, 1996). The measured value of α in this case is approximately 9.5 μm and 2.8 μm under 1-p and 2-p excitation, respectively. The lower resolution under 1-p excitation is caused by the fact that spherical aberration resulting from the refractive index mismatch between the cover glass of the cell and water under 1-p excitation is stronger than that under 2-p excitation (Ke and Gu, 1998; Török et al., 1996). When the distilled water is replaced by polystyrene beads of diameter 0.202 μm, image resolution under 1-p and 2-p excitation becomes approximately 147 μm and 17 μm, respectively (Fig. 1b). This result shows a reduction of resolution by 15 and 6 times, respectively, compared with that in Figure 1a.

To further demonstrate the effect of multiple scattering on image resolution under 1-p and 2-p excitation, we show

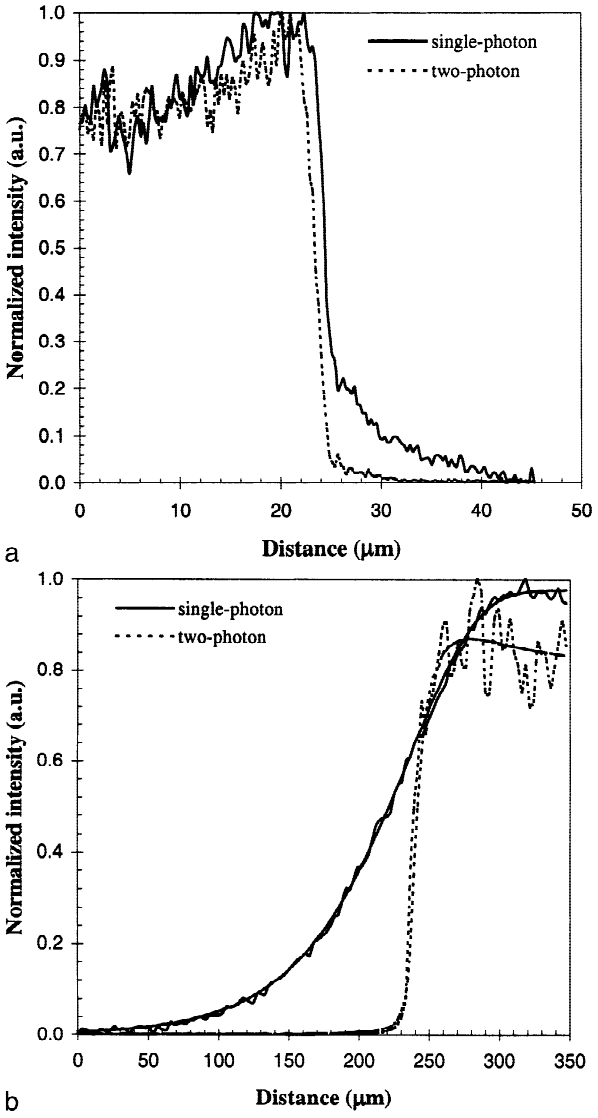

Figure 1. Cross-section of the image intensity of a bar under 1-p and 2-p excitation. **a:** A bar embedded in a cell (d = 100 μm) consisting of water. **b:** A bar embedded in a cell (d = 100 μm) consisting of polystyrene beads of diameter 0.202 μm (thin solid curves are the fitted curves).

in Figure 2 the image resolution α and the optical thickness n (n is defined as the cell thickness divided by the SMFP length) as a function of the cell thickness d for four types of scattering particles. It can be clearly seen from Figure 2 that the image resolution under 1-p excitation deteriorates significantly faster than that under 2-p excitation as d increases. For example, compared with those at d = 0, the image resolutions under 1-p and 2-p excitation are decreased by approximately 12 and 5 times at d = 300 μm, 24 and 8 times at d = 150 μm, 26 and 12 times at d = 50 μm, and 8 and 3 times at d = 25 μm in Figures 2a–d, respectively. This result is caused by the fact that the SMFP length under 1-p excitation is smaller than that under 2-p excitation. A smaller particle size leads to a larger difference of the SMFP length between 1-p and 2-p excitation. It is therefore concluded that 2-p excitation can significantly improve image resolution by reducing the number of scattering events experienced by the excitation beam, in particular when scattering particles are small.

In the case of 1-p excitation, the α value can be measured only when the cell thickness d is increased up to 150 μm, 50 μm, and 25 μm in Figures 2b–d, respectively. This phenomenon is because the corresponding images significantly blur although signal strength is still detectable. However, the cell thickness d under 2-p excitation is limited to 75 μm and 50 μm in Figures 2c and d, respectively, mainly because the corresponding 2-p fluorescence signal is too weak to be detectable as the SMFP length decreases with the size of scattering particles (see Table 1). Thus, image quality under 1-p excitation is mainly limited by the degradation of image resolution caused by multiple scattering, while image quality under 2-p excitation is mainly limited by the degradation of signal strength/contrast. This conclusion is further demonstrated in Figure 3. Figures 3a and b are the 1-p and 2-p fluorescence images of a bar embedded in a given turbid sample, which show the stronger degradation of the image resolution in the former case. When the thickness of the turbid sample is increased by twice, images under 1-p excitation exhibit no sharpness at the edge of the bar (not shown here). But the edge of the bar still can be seen with a low contrast under 2-p excitation (Fig. 3c).

The image resolution achievable under 2-p excitation is determined by the number of scattering events experienced by the excitation beam, which leads to three regions shown by Figure 2. The first region is demonstrated by Figure 2a in which multiple scattering is not so strong because the optical thickness for the excitation beam is less than 1. In this case, 2-p excitation is produced mainly by unscattered photons of the illumination beam. As a result, the image resolution achievable is close to that measured without turbid media. As the number of scattering events experienced by the excitation beam increases, the unscattered component of the illumination beam significantly reduces. Therefore the value of α deteriorates quickly due to the quadratic dependence of the 2-p fluorescence on the incident power (no such a feature under 1-p excitation). This feature is illustrated in Figure 2b which shows that α increases at a

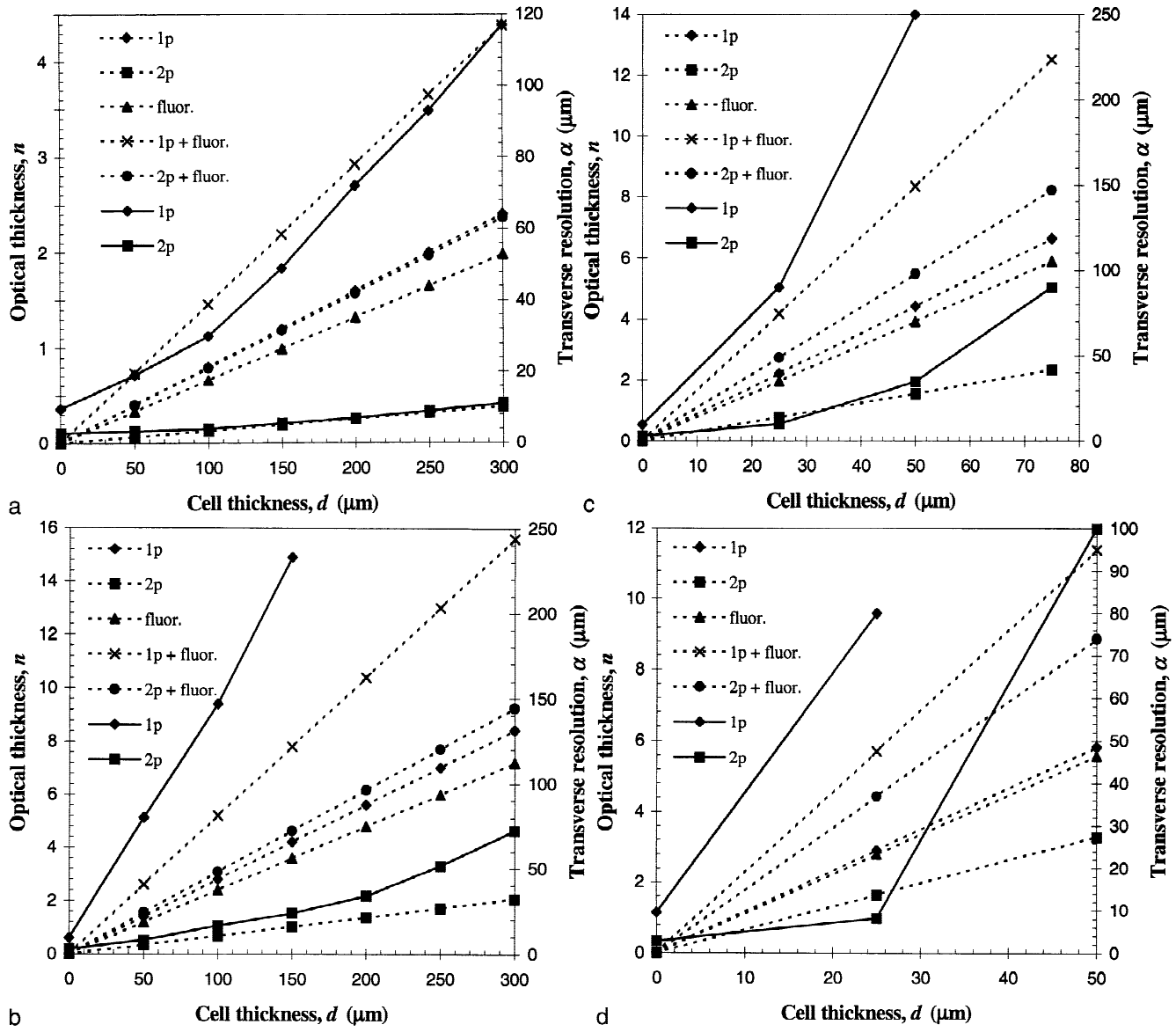

Figure 2. Optical thickness n under 1-p and 2-p excitation (dashed) and image resolution α (solid) as a function of the cell thickness d. **a:** A turbid medium including beads of diameter 0.107 μm. **b:** A turbid medium including beads of diameter 0.202 μm. **c:** A turbid medium including beads of diameter 0.0.48 μm. **d:** A turbid medium including beads of diameter 1.056 μm.

steady rate until about 200 μm. After a depth of 200 μm, α increases quickly, indicating that multiple scattering of the illumination beam is strong in this region (the second region). A similar trend is seen in Figure 2c. If the excitation beam experiences more scattering events, scattered photons of the illumination beam become dominant, leading to a significant reduction in image resolution. This third region can be seen from Figure 2d when d is larger than 25 μm.

Another major physical difference among Figures 2a–d is the scattering anisotropy of the turbid media under inspection (see Table 1). As the scattering particle size increases, a larger proportion of scattered photons propagate in the forward (illumination) direction due to their larger g value. Thus, the image quality achievable for a given optical thickness n of the illumination beam should be improved as the size of scattering particle size increases. It is seen from Figure 2 that at a given number of scattering events of the illumination beam, the image resolution α achievable under 2-p excitation is improved when a turbid medium consisting of larger scattering particles (Fig. 2d) is used. However, such an improvement under 1-p excitation is not pronounced because of the poorer resolution which leads to less accurate measurements.

Finally, it should be pointed out that spherical aberra-

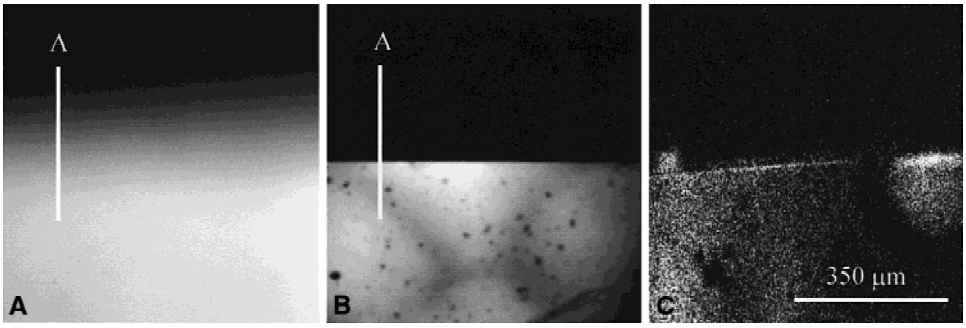

Figure 3. Images of a bar embedded within a turbid medium including polystyrene beads of diameter 0.202 μm. The intensity cross-sections at position A and the corresponding fitted curves are shown in Figure 1b. **a:** 1-p excitation and thickness d = 100 μm. **b:** 2-p excitation and thickness d = 100 μm. **c:** 2-p excitation and thickness d = 300 μm.

tion caused by the mismatch of refractive indices between the cover glass of the cell and water can also lead to a reduction in image resolution. This type of aberration can broaden the point spread function of the imaging objective by a factor of 2–3 and reduce the intensity at the diffraction focus by approximately 90% for a water layer of thickness 200 μm (Ke and Gu, 1988). However, these degradations are negligible compared with those caused by multiple scattering.

Conclusions

In conclusion, it has been demonstrated that 2-p excitation can significantly improve the penetration depth and image quality achievable when an object is embedded in turbid media. Image quality under 2-p excitation may be appreciably degraded as long as the scattered photons in the excitation beam become dominant. The image quality achieved by a given detector is limited by the 2-p fluorescence strength rather than by the image resolution. However, the degradation in image resolution is a main limiting factor in obtaining high quality images in the case of 1-p excitation.

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