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Monte Carlo simulation of multiphoton fluorescence microscopic imaging through inhomogeneous tissuelike turbid media

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Abstract. Image resolution and signal level in fluorescence microscopy through inhomogeneous turbid media consisting of scatterers of multiple sizes under single- (1p), two- (2p), and three-photon (3p) excitation have been investigated based on a modified Monte Carlo model. The effects of the size distribution and the concentration distribution of scattering particles are explored. Simulation results reveal that the size and the concentration distribution both have an impact on image formation in media consisting of small particles and that 3p excitation has the most significant impact. In media with scatterers of a large size, both size and concentration distributions lead to a slight effect. Image formation in a mixed medium containing small and large scattering particles is more affected by the large particles. [DOI: 10.1117/1.1577116]

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1 Introduction

Since its first demonstration by Denk et al. in 1990,[1] two-photon (2p) fluorescence microscopic imaging has been widely applied as a useful tool in biomedical studies.[2–10] In 2p excitation, the scattered photons are too dilute to cause simultaneous absorption of two photons within the focal region, which automatically avoids off-focus excitation and provides three-dimensional optical sectioning and resolution equivalent to that of a confocal microscope.[1–3] Another advantage of 2p excitation is based on the utilization of an infrared laser beam for excitation, which highly reduces Rayleigh scattering in tissue media, in which case the size of scatterers in tissue is smaller than the illumination wavelength. Since the strength of Rayleigh scattering is inversely proportional to the fourth power of the illumination wavelength, it has been expected that three-photon (3p) excitation could further reduce the effect of multiple scattering in tissue media[11,12] and result in great penetration depth. However, biological tissue is usually composed of scatterers varying from 0.1 μm to a few micrometers in size.[13,14] Thus in this situation, the dominant effect caused by these scatterers is Mie scattering rather than Rayleigh scattering.

The effect of Mie scattering on multiphoton excitation in turbid media has been studied using Monte Carlo simulation.[15–20] So far, the model for turbid media considered in the current Monte Carlo simulation has been based on a homogeneous turbid media structure in which the size of all the scatterers is the same.[15–18,20] However, biological tissue usually exhibits a complex inhomogeneous structure; the basic components of biological tissue, cells, range widely in size, from nanometers to tens of micrometers, and numerous different sizes of scattering organelles exist within the cells.[21] For example, most animal cells range from 10 to 30 μm in size and the nuclei, the largest scattering organelles found in cells, have sizes ranging between 3 and 10 μm. Mitochondria are small organelles with sizes ranging from 0.5 to 1.5 μm. Other smaller cell components include endoplasmic reticulum (0.2 to 1 μm), lysomes (0.2 to 0.5 μm), and peroxisomes (0.2 to 0.5 μm).[22] Therefore, it is necessary to modify the current Monte Carlo simulation model to investigate image formation in a tissuelike turbid medium that is composed of scattering particles of several sizes.

In this paper, a modified Monte Carlo simulation model considering inhomogeneous turbid media with scatterers of several sizes is established for studying multiphoton fluorescence microscopy. The paper is organized as follows. In Sec. 2, a modified Monte Carlo simulation model is described to deal with the scattering features from a turbid medium with particles of several sizes. In Sec. 3, the effect of the size distribution of scatterers is investigated in a medium with a uniform distribution of concentration. For comparison, the effect of the Gaussian concentration distribution and the combined distribution including a mixture of large and small scatterers is explored in Sec. 4. The discussion is presented in Sec. 5 and a conclusion is given in Sec. 6.

2 Modified Monte Carlo Simulation Model for a Turbid Medium of Multisized Scatterers

The schematic diagram for imaging through turbid media in a reflection-mode fluorescence microscope and the derivation of an effective point-spread function (EPSF) at a given focal depth under 1p, 2p, and 3p excitation have been reported elsewhere.[17,20,23] The focal depth, f_d, is defined as the distance between the medium's surface and the focal plane of an imaging objective.

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In this paper, a single-layer inhomogeneous turbid medium consisting of n types of spherical scatterers is considered. Each type of scatterer has a given size (diameter) ρ_i and concentration c_i. A scattering mean-free-path length (l_i) for each type of scatterer is determined by its concentration and its corresponding scattering cross-section (σ_{si}), based on Mie theory[24]:

$$l_i = 1/(c_i \sigma_{si}). \tag{1}$$

To determine the length of the scattering step between two consecutive scattering events of a photon in a turbid medium with multisized scattering particles, we first independently calculate the scattering step length (s_i) from each type of particle according to the following equation:

$$s_i = -l_i \ln(\xi) \quad (i = 1,2,...n), \tag{2}$$

where $0<\xi<1$ is a randomly produced uniform distribution number. Then the shortest step length (s_m) is taken to be the length within which a photon propagates freely. The anisotropy value (g_m) corresponding to the m'th type of particle, which is also calculated based on Mie theory,[24] is used to determine the scattering direction of the photon. The accuracy of this method was verified by our previous model when there is only one type of particle.[17,20]

For understanding image performance in a turbid medium of multisized scattering particles, a parameter, the effective mean-free-path length (l'), is introduced as a measure of the randomness in such an inhomogeneous medium:

$$1/l' = \sum_{i=1}^{n} 1/l_i. \tag{3}$$

The effective mean-free-path length weights the contributions of scattering cross-sections from different types of particles to the scattering features of the turbid medium. It is a parameter analogous to the mean-free-path length (l) in a homogeneous medium that has scattering particles of one size.

The parameters used in the Monte Carlo simulation are introduced as follows: 10^7 illumination photons are used to ensure the accuracy of simulation results under 1p, 2p, and 3p excitation. The numerical aperture of the objective is 0.25, the same as in our previous study.[20] It is assumed that the wavelength of the excitation beam, λ_{ex}, under 1p, 2p, and 3p excitation is 400, 800, and 1200 nm, respectively, and the fluorescence wavelength, λ_{fluo}, is 400 nm in the three cases.

According to the size distribution of small organelles and nuclei,[22] in this paper three groups of inhomogeneous turbid media are considered for understanding image formation. Groups S and L, which are used to investigate the scattering features from small organelles and large nuclei in cells, contain nine types of scattering particles. Their diameter (ρ) ranges from 0.1 to 0.9 μm and from 2.6 to 3.4 μm, respectively, with a diameter interval of 0.1 μm; the mean size of these two groups is 0.5 and 3.0 μm, respectively. The third group of the turbid media (M) is a mixture of groups S and L to simulate the situation where small scattering organelles and large nuclei both exist. Based on Mie theory,[24] the corresponding anisotropy value (g_i) and scattering cross-section (σ_{si}) of each type of particle under 1p, 2p, and 3p excitation are shown in Table 1.

For a comprehensive understanding of the effect of the particle concentration c_i, two types of concentration distributions, a uniform and a Gaussian distribution, are investigated and represented by U or G. All concentration distributions discussed here are centrally symmetrical through the mean particle size. Each turbid medium is labeled by two letters followed by two numerals. The first letter represents the type of particle group, while the second letter denotes the type of concentration distribution. The two numerals correspond to a width parameter δ. The width parameter in the medium with a uniform distribution is $\delta_p/2$ ($\delta_p = \rho_{max} - \rho_{min}$) where ρ_{max} and ρ_{min} are the maximum and minium particle sizes, respectively. In the medium with a Gaussian distribution, the width parameter is defined as the full width at half maximum of the concentration distribution. For example, the SG01 medium contains a small group of particles with a Gaussian distribution, and δ=0.1 μm.

In order to observe the scattering features of different concentrations, it is assumed that the averaged total geometrical cross-section (σ) is constant in all media, which can be mathematically expressed as

$$\int \pi(\rho/2)^2 c(\rho) \mathrm{d}\rho = \pi(\rho_0/2)^2 c_0, \tag{4}$$

where ρ is the diameter for a particle type existing in an inhomogeneous medium and $c(\rho)$ is the corresponding concentration. Here ρ_0 refers to the scattering particle size in a homogeneous medium with a concentration c_0. In our calculation $\rho_0 = 0.5\ \mu$m and $c_0 = 0.6/\mu\mathrm{m}^3$ for the uniform S group. Accordingly, in the uniform L group, $\rho_0 = 3\ \mu$m and $c_0 = 0.0167/\mu\mathrm{m}^3$.

According to the concentration distributions and the scattering cross-section at different excitation wavelengths (Table 1), the effective mean-free-path lengths (l') of the various inhomogeneous media under 1p, 2p, and 3p excitation can be calculated by Eqs. (1) and (3) and are summarized in Table 2.

3 Effect of Size Distribution on Multiphoton Fluorescence Microscopy

To investigate the effect of size distributions on image performance under multiphoton excitation, the image intensity $I(x,y)$ of a thin object in an imaging system is calculated using the convolution of the object function $O(x,y)$ and an effective point-spread function $h(x,y)$.[22]

$$I(x,y) = \int \int_{-\infty}^{\infty} h(x',y') O(x-x', y-y') \mathrm{d}x' \mathrm{d}y'. \tag{5}$$

Once the image intensity distribution of the object is obtained, the transverse resolution Γ is characterized by the distance between the 90 and 10% intensity points from the image intensity of the sharp edge scanned in the x direction. The signal level (η) is defined as the number of fluorescent photons collected by the detector and normalized by that number when no scattering exists. Both transverse resolution and signal level are then used to measure the performance of an imaging system that includes a turbid medium.[20,22]

The EPSF indicates the performance of an imaging system that includes a turbid medium.[20,22] As an example, EPSFs for

Table 1 Scattering parameters (σ_s, g) of the small and large groups of scattering particles under 1p, 2p, and 3p excitation.

ρ (μm)		0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
400 nm (1p excitation) (Fluorescence)	σ_{si} (μm^2)	0.000296	0.0081	0.0483	0.155	0.36	0.686	1.132	1.678	2.28
	g_i	0.193	0.685	0.804	0.864	0.894	0.916	0.924	0.928	0.929
800 nm (2p excitation)	σ_{si} (μm^2)	0.000023	0.00128	0.00877	0.0324	0.0907	0.193	0.366	0.619	0.971
	g_i	0.047	0.193	0.450	0.685	0.739	0.804	0.844	0.864	0.885
1200 nm (3p excitation)	σ_{si} (μm^2)	0.0000047	0.000277	0.00266	0.0114	0.0317	0.073	0.150	0.268	0.435
	g_i	0.0209	0.084	0.193	0.353	0.547	0.685	0.727	0.757	0.804
ρ (μm)		2.6	2.7	2.8	2.9	3.0	3.1	3.2	3.3	3.4
400 nm (1p excitation) (Fluorescence)	σ_{si} (μm^2)	14.28	15.1	15.65	16.16	16.62	16.56	16.23	15.98	16.44
	g_i	0.889	0.896	0.892	0.877	0.863	0.863	0.870	0.877	0.872
800 nm (2p excitation)	σ_{si} (μm^2)	16.45	16.6	16.88	16.91	16.79	16.92	16.56	16.85	16.71
	g_i	0.906	0.900	0.892	0.883	0.874	0.863	0.852	0.844	0.829
1200 nm (3p excitation)	σ_{si} (μm^2)	18.73	20.50	22.37	24.21	25.88	27.68	29.23	30.73	32.20
	g_i	0.930	0.929	0.930	0.930	0.929	0.927	0.926	0.925	0.922

Table 2 Effective mean-free-path lengths (l') of turbid media investigated under 1p, 2p, and 3p excitation.

		l' (μm)					l' (μm)		
Type of medium		400 nm (1p excitation) (Fluorescence)	800 nm (2p excitation)	1200 nm (3p excitation)	Type of medium		400 nm (1p excitation) (Fluorescence)	800 nm (2p excitation)	1200 nm (3p excitation)
SU	SU00/ SG00	4.6296	18.3756	52.5762	LU	LU00/ LG00	3.6029	3.5664	2.3138
	SU01	4.2699	16.2234	44.1706		LU01	3.6850	3.5918	2.3379
	SU02	3.7832	13.0401	33.5206		LU02	3.6863	3.5618	2.3143
	SU03	3.3224	10.3066	25.1631		LU03	3.7573	3.5907	2.3363
	SU04	2.9837	8.2988	19.4841		LU04	3.8000	3.6071	2.3473
SG	SG01	4.5736	18.0067	51.0311	LG	LG01	3.5956	3.5501	2.3044
	SG02	4.3182	16.2384	44.0992		LG02	3.6360	3.5524	2.3098
	SG03	3.9779	13.9597	36.3106		LG03	3.7065	3.5959	2.3383
	SG04	3.6824	12.0424	30.2078		LG04	3.6324	3.5029	2.2781
M	SG04+ LU02	1.8422	2.7488	2.1496					

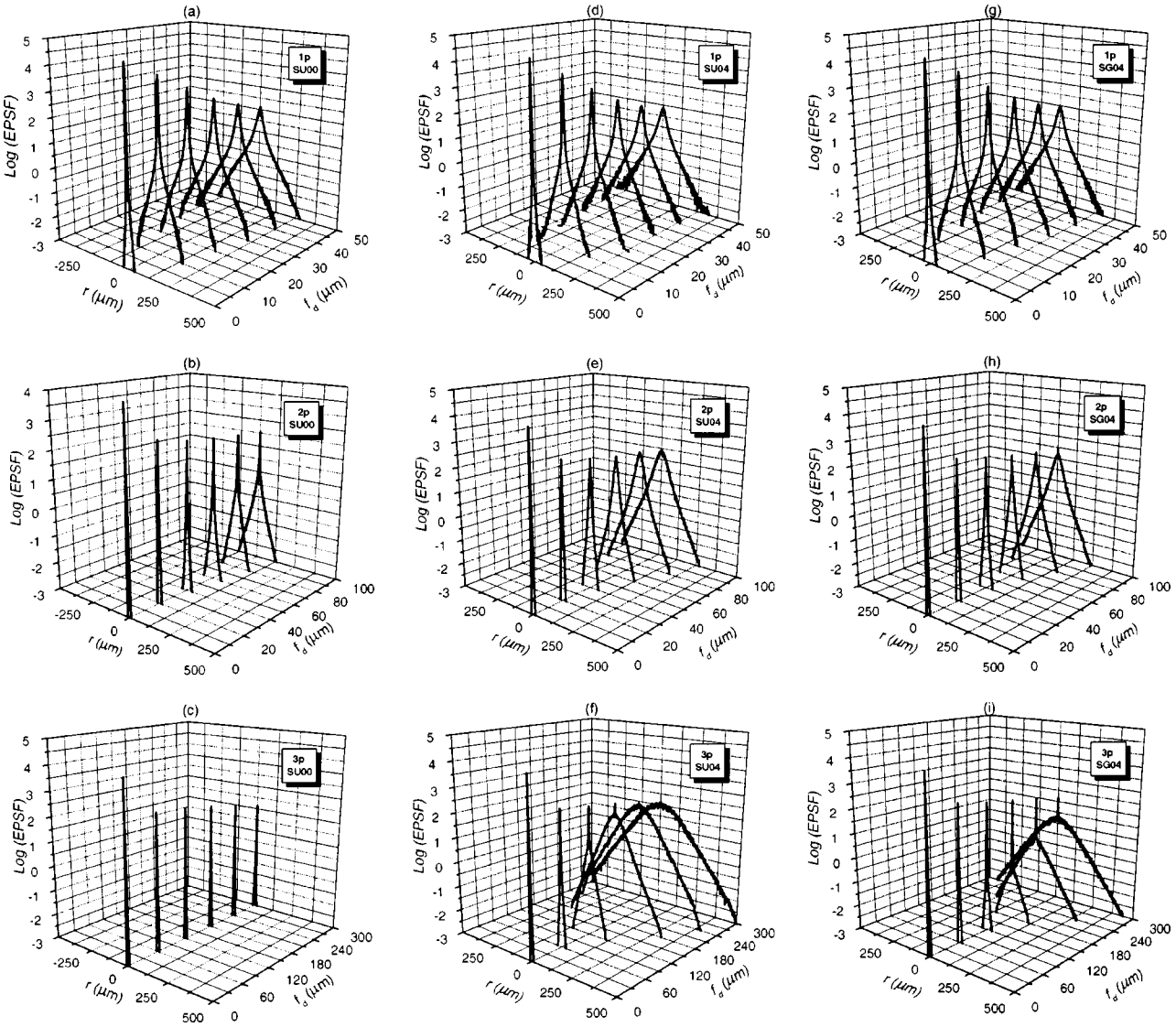

Fig. 1 EPSFs for fluorescence imaging at different focal depths in SU00, SU04, and SG04 turbid media under 1p, 2p, and 3p excitation.

turbid media SU00, SU04, and SG04 at different focal depths under multiphoton excitation are shown in Fig. 1. Here r is the radial distance in the focal plane. The sharp peak shown in Fig. 1 indicates the existence of ballistic and less-scattering snake photons. It can be seen that as the focal depth increases, the EPSF becomes broad and the peak value decreases. This is because as the focal depth increases, the scattering events increase, which leads to the broadening of the EPSF. Meanwhile, owing to strong scattering, the number of the ballistic and snake photons that undergo few scattering events around the center of the focus region decreases. As a result, the peak value falls.

A comparison of the EPSFs in SU00 and SU04 and SG04 media under 1p, 2p, and 3p excitation shows that at the given focal depth, the EPSF under 3p excitation is much narrower than that under 1p excitation. As the focal depth increases, the reduction rate of the peak value under multiphoton excitation (2p and 3p excitation) becomes slower than that under 1p excitation. This feature indicates that multiphoton excitation can give higher resolution at a given focal depth and has a better penetration ability than 1p excitation. Under the same excitation type and a given focal depth, the EPSFs are the narrowest presented in the homogeneous SU00 media.

The transverse image resolution and the signal level in a series of SU media under 1p, 2p, and 3p excitation are shown in Fig. 2. Although the total geometrical cross-sections of the distributed-size turbid media are equal, optical microscopic image formation in those media is affected differently owing to the different scattering features of scatterers of various sizes. The more that particles around a mean size of 0.5 μm are included, the worse the image performance on resolution and signal level. However, under 1p excitation, the effect of the size distribution is not as obvious as that under multiphoton excitation. Three-photon excitation produces the most visible effect on transverse resolution and signal level. This situation may be explained by the effective mean-free-path length introduced in this paper. Table 2 clearly shows that in the medium that has a δ value of 0.4 μm (SU04), l' has the shortest value in three excitation cases and in that under 3p

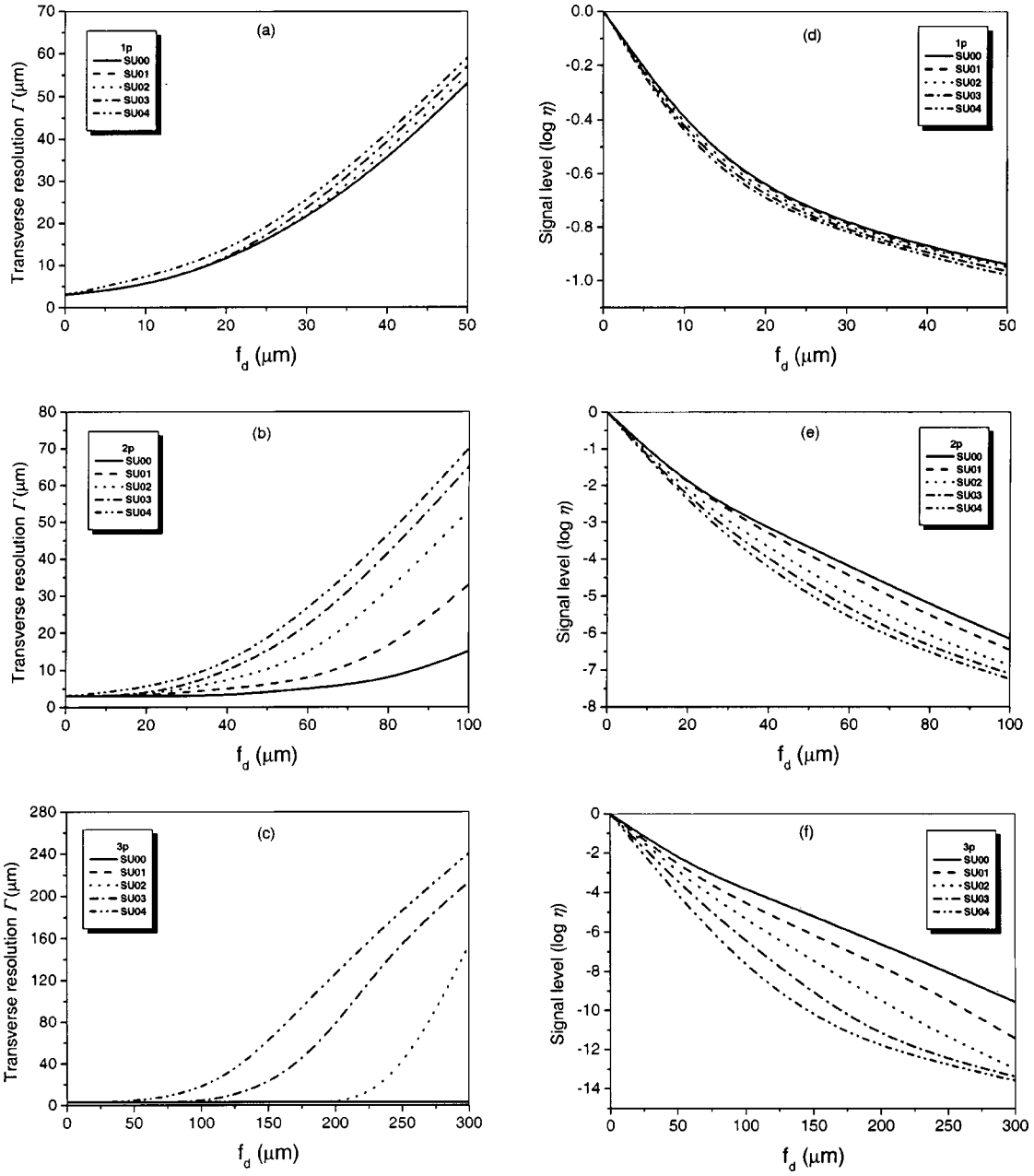

Fig. 2 Transverse image resolution (a–c) and signal level (d–f) as a function of the focal depth in the SU media under 1p, 2p, and 3p excitation.

excitation, the difference in l' between different types of media is the most obvious.

Figure 3 shows the transverse resolution and signal level in a series of LU media under 1p, 2p, and 3p excitation. Unlike the situation in the SU media, the difference in the transverse resolution and signal level among different types of media is not significant under three types of excitation. Under 3p excitation, the difference is even less than that under 1p excitation, as can be seen from the value of l' in Table 2. Furthermore it can be seen that the LU00 medium presents the worst imaging performance in particular under 1p excitation. This phenomenon is expected from the l' value in Table 2.

4 Effect of the Concentration Distribution on Multiphoton Fluorescence Microscopy

In a turbid medium with particles of several sizes, the concentration distribution of scatterers is also important. Therefore, in this section, a Gaussian concentration distribution and a mixed uniform and Gaussian concentration distribution will be investigated.

Figure 4 shows the transverse resolution and signal level in the SG media. Compared with the SU media (Fig. 2), the transverse resolution and signal level under multiphoton excitation (2p and 3p excitation) are better in the SG media. However, under 1p excitation, the difference in transverse resolu-

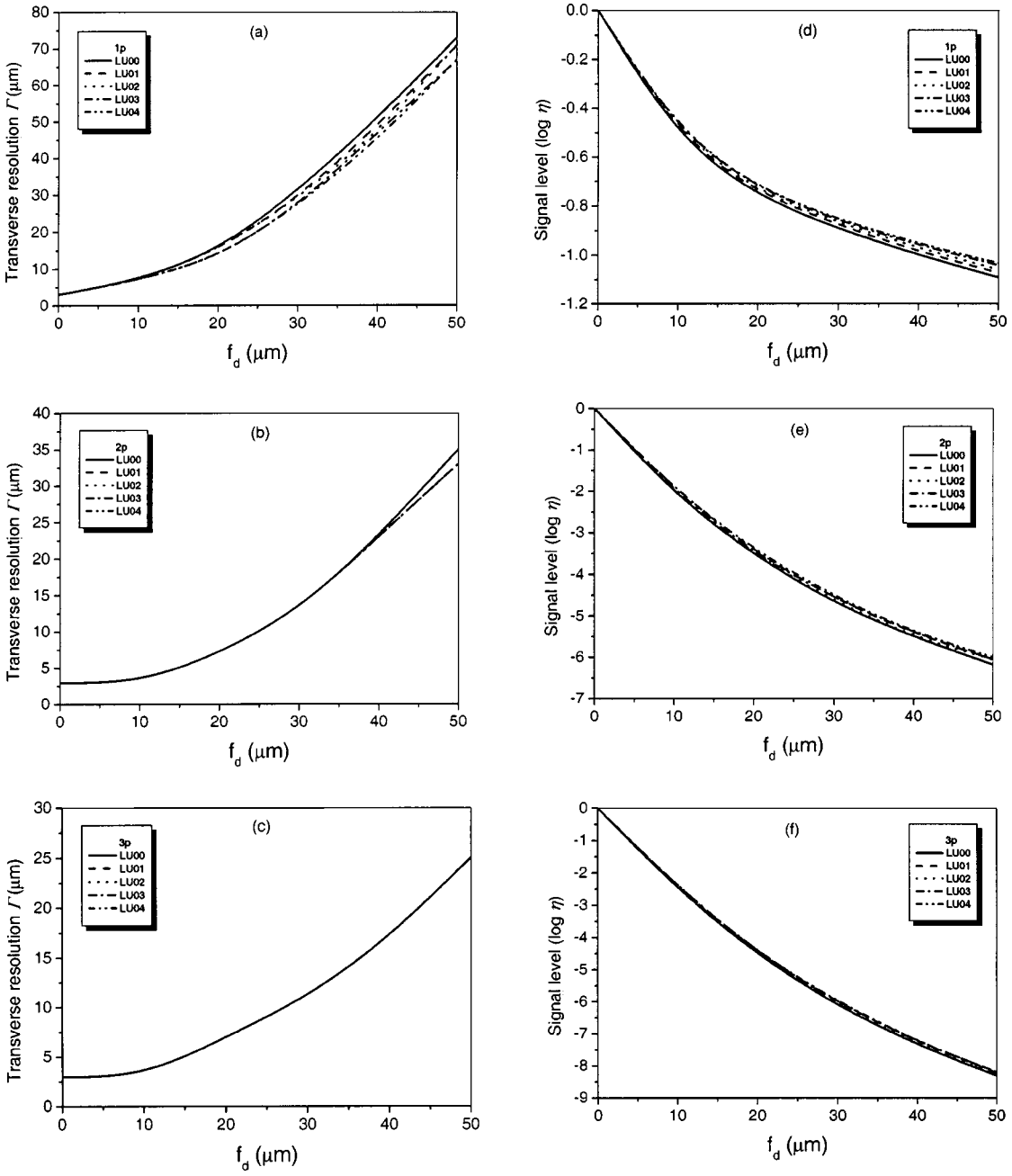

Fig. 3 Transverse image resolution (a–c) and signal level (d–f) as a function of focal depth in the LU media under 1p, 2p, and 3p excitation.

tion and signal level is not obvious. Another comparison of transverse resolution between SG and SU media shows that under 3p excitation, two types of media—SG01 and SG02—maintain the diffraction-limited image resolution within a depth of 300 μm. However, in the SU media, only SU01 can keep the diffraction-limited image resolution within this region. An explanation can be obtained from the fact that in the SG media l' is larger than in the SU media under multiphoton excitation but is only slightly larger under 1p excitation. This feature is caused by the suppression of the effect of the large particles in the case of the Gaussian distribution. This feature can be also seen in the EPSFs shown in Fig. 1. At the same focal depth, the EPSF in SG04 is narrower than that in SU04 under multiphoton excitations, while the difference is not obvious under 1p excitation.

The same is true for the L group media with a Gaussian concentration distribution, the transverse resolution and signal levels of which are shown in Fig. 5. There is little difference in the transverse resolution and the signal level in the LG and LU media under 1p, 2p, and 3p excitation.

A mixed medium is investigated to simulate the combined effect of uniform and Gaussian distributions. In this simulation, it is supposed that there is a diverse distribution of particles of small sizes and a relatively concentrated distribution of particles of large sizes. This condition corresponds to the situation in cells where small scatterers (such as small or-

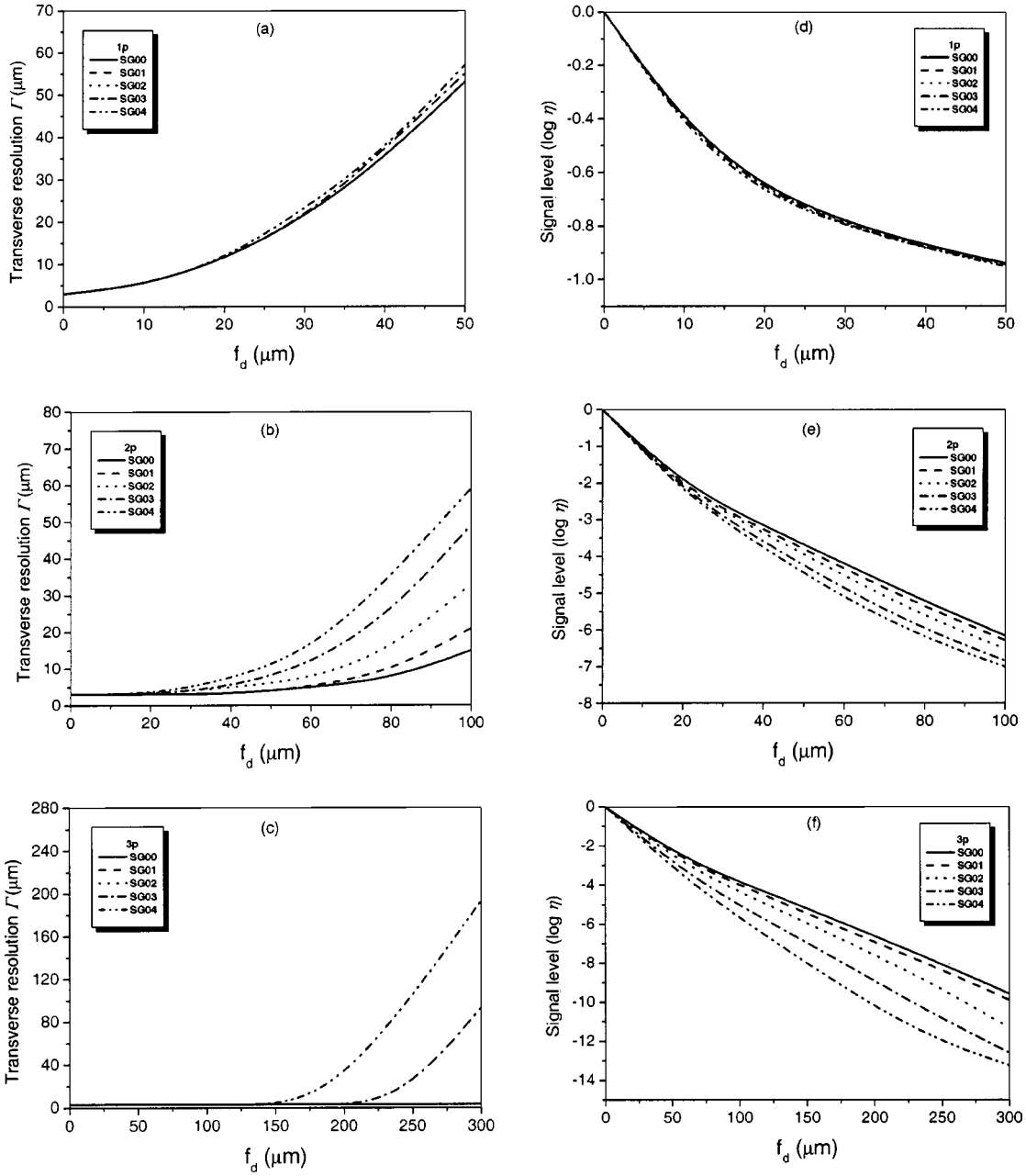

Fig. 4 Transverse image resolution (a–c) and signal level (d–f) as a function of focal depth in the SG media under 1p, 2p, and 3p excitation.

ganelles) distribute diversely while the size of scattering particles such as nuclei has a relatively small variation. It can be seen from Figs. 3 and 5 that for the L group, the imaging performance with the uniform and Gaussian distribution is not significant and the effect of the size distribution is not pronounced. Therefore, in the mixed medium M, we arbitrarily choose LU02 to be a simulation medium for nuclei. On the other hand, SG04 is selected to simulate the maximum effect of the size distribution of small scatterers. However, in order to keep the equality of the total geometrical cross, M has half of the concentration values for each of its corresponding scatterers compared with those in the SG04 and LU02 media.

Figure 6 shows the transverse resolution of the image and the signal level under 1p, 2p, and 3p excitation in medium M. For comparison, the transverse resolution and signal level in the SG04 and LU02 media are also given in the same plots. It can be seen that image performance in medium M (either transverse resolution or signal level) falls in between two extreme cases in which the turbid medium consists of either small or large particles, and the image performance is more affected by the LU02 medium than the SG04 medium under three types of excitation.

5 Discussion

The simulation results show that image formation in an inhomogeneous turbid medium that has scattering particles of

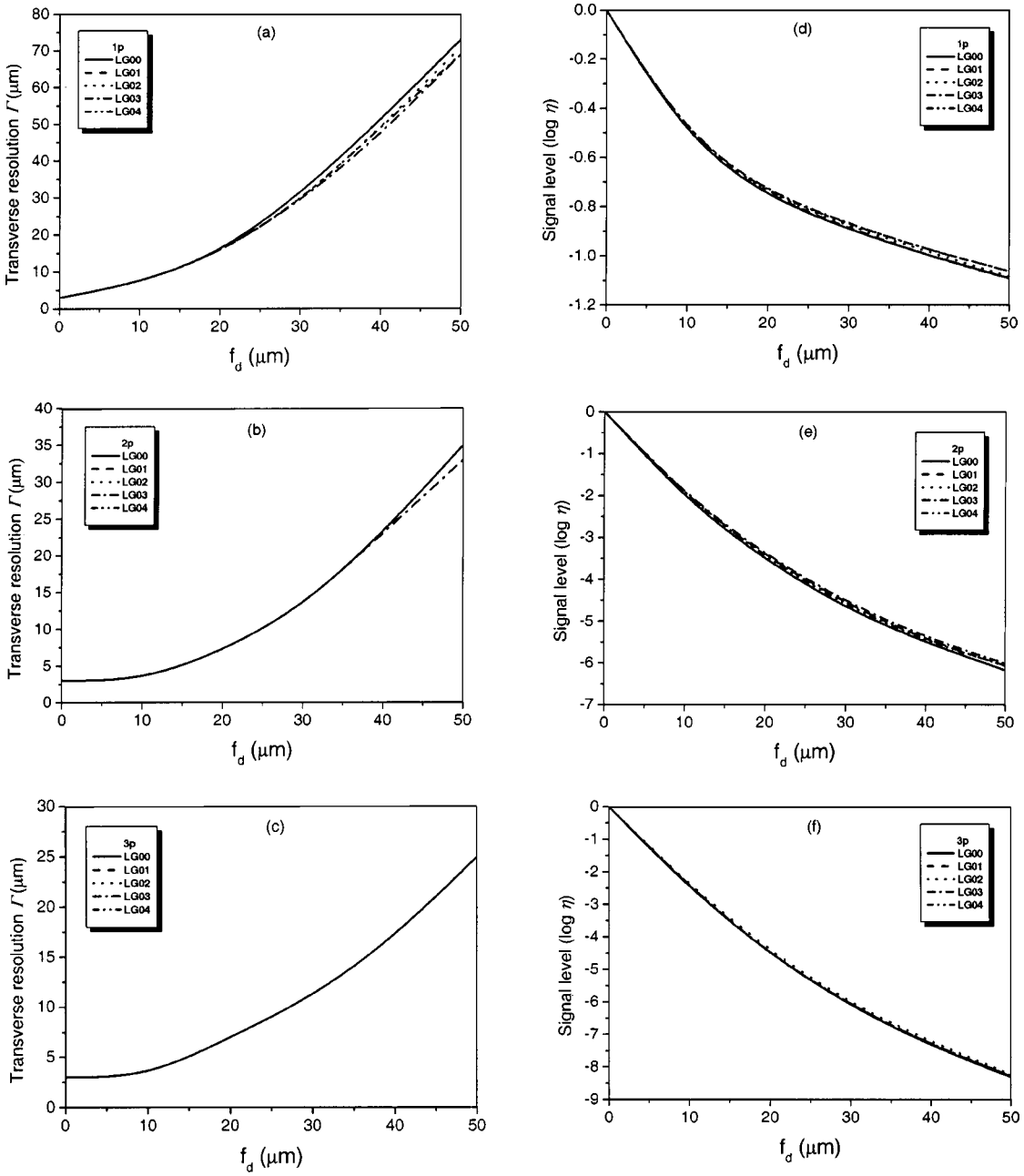

Fig. 5 Transverse image resolution (a–c) and signal level (d–f) as a function of focal depth in the LG media under 1p, 2p, and 3p excitation.

multiple sizes is more complicated than that in a homogeneous medium containing scattering particles of a single size.[17,20]

First, the mean-free-path length (l) of each type of scattering particle in an inhomogeneous turbid medium may not give a clear indication of the medium's properties for image formation. Accordingly, a concept of the effective mean-free-path length (l') is introduced, which is a comprehensive description of the scattering feature (scattering length) from all types of particles in the medium. Image performance can be understood by the l' value, which is similar to the l value in a homogeneous medium.

Second, the effect of the size and concentration distributions on image performance is significant. The S and L media exhibit dramatically different features of image formation; the variation of the size distribution in the L media has a slight impact on image resolution and signal level under three types of excitation, while the corresponding influence in the S media is obvious, especially under multiphoton excitation. It is also evident that at a given focal depth, imaging in the S media exhibits better transverse resolution and signal level than that in the L media.

These phenomena can be explained from the relationship of the scattering efficiency Q and the anisotropy g value to a scattering parameter (a/λ) (Fig. 7). The scattering efficiency Q is defined as the ratio of the scattering cross-section (σ_s) to the geometrical cross-section (σ), and a is the radius size of a scattering particle. The smaller scattering efficiency implies a

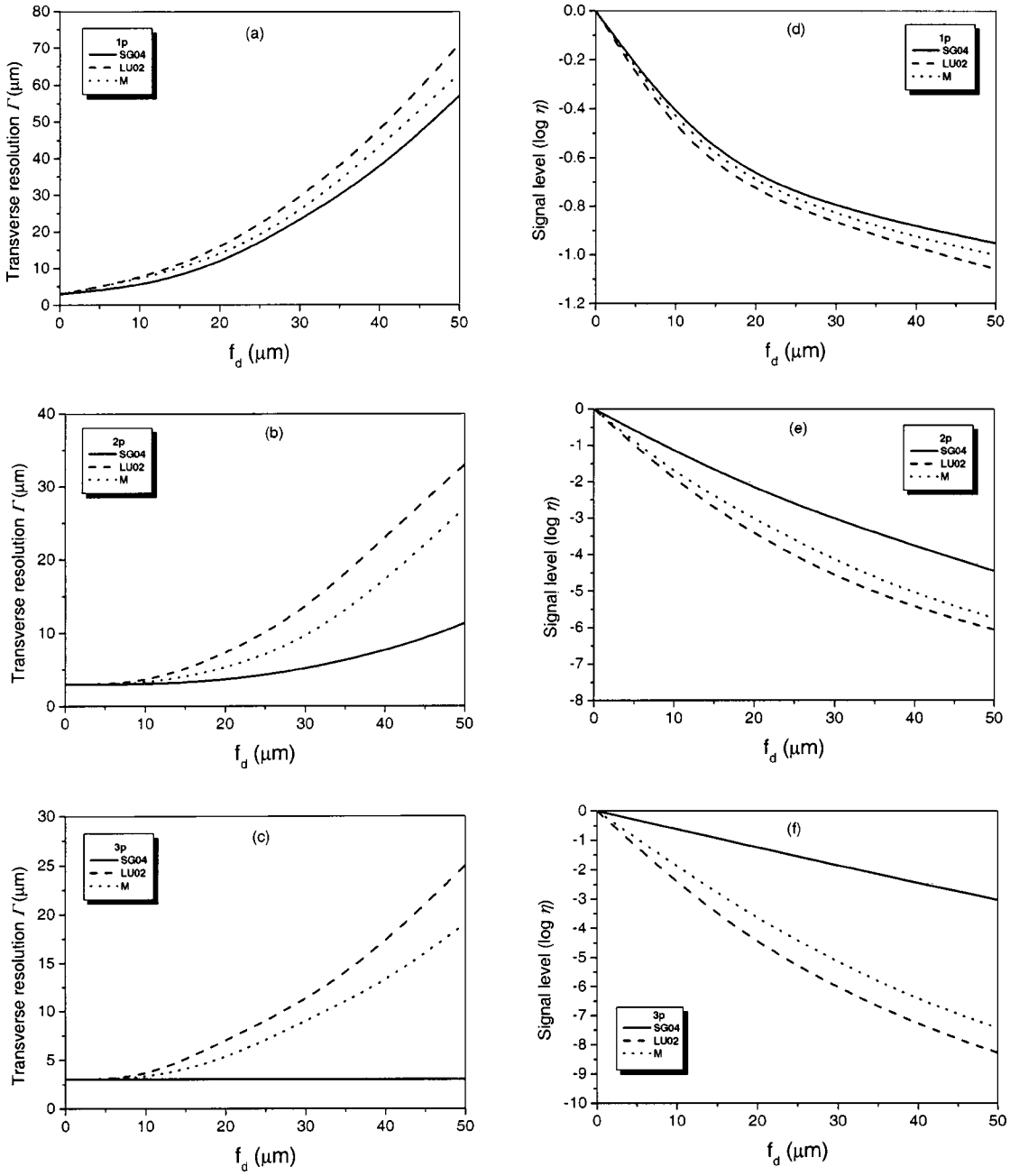

Fig. 6 Transverse image resolution (a–c) and signal level (d–f) as a function of focal depth in the mixed medium under 1p, 2p, and 3p excitation.

reduction of multiple scattering events, which leads to high image resolution and signal level. In our case, the S media include particles that have relative sizes a/λ within ranges from 0.125 to 1.125, 0.0625 to 0.5625, and 0.0417 to 0.375 under 1p, 2p, and 3p excitation, respectively. The L media include the relative particle sizes within ranges from 3.25 to 4.25, 1.625 to 2.125, and 1.083 to 1.417 under 1p, 2p, and 3p excitation, respectively. Thus the scattering Q changes significantly as a/λ falls within the range of 0.0417 to 1.125 in the S media and shows a slight variation when a/λ falls within the range of 1.083 to 4.25 in the L media, as indicated in Fig. 7. This feature provides an understanding of the significant influence of the scattering features in the S media. In the S media, the Q value of the largest particles (0.9 μm) is about 100, 500, and 1000 times that of the smallest particles (0.1 μm) under 1p, 2p, and 3p excitation, respectively. This effect results in the most significant impact in 3p excitation on the image performance.

6 Conclusion

Image resolution and signal level in fluorescence microscopy through turbid media with scattering particles of multiple sizes under 1p, 2p, and 3p excitation have been explored through Monte Carlo simulation. The dependence of resolution and signal level on the focal depths in three groups of inhomogeneous turbid media (S, L, and M) has been demonstrated.

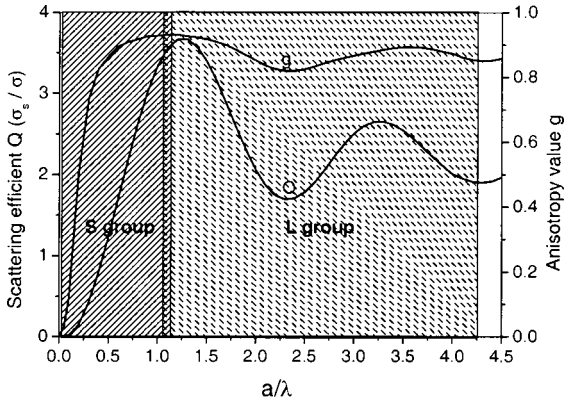

Fig. 7 Scattering efficiency Q and anisotropy value g as a function of the scattering parameter a/λ. The refractive indices of scatterers (spherical particles) and the immersion medium (water) are 1.59 and 1.33, respectively.

The results show that in the S media, the size distribution of the scatterers has a significant effect on image formation; the broader the distribution, the worse the image resolution and signal level. At a given depth, 3p excitation shows the most significant difference in image performance in the different types of media compared with 1p and 2p excitation. The image resolution and signal level in the media with a Gaussian concentration distribution are better than those in the media with a uniform distribution. However, the impact of the size and concentration distributions in the L media is not significant.

The combined effect of the uniform and Gaussian concentration distributions for the large and small groups of scatterers indicates that image performance is affected more by the large group of particles, for example, the nulei distributed in tissue.

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