Time multiplexing and parallelization in multifocal multiphoton microscopy

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Received September 2, 1999; accepted February 23, 2000

We investigate the imaging properties of high-aperture multifocal multiphoton microscopy on the basis of diffractive theory. Particular emphasis is placed on the relationship between the sectioning property and the distance between individual foci. Our results establish a relationship between the degree of parallelization and the axial resolution for both two- and three-photon excitation. In addition, we show quantitatively that if a matrix of temporal delays is inserted between the individual foci, it is, for the first time to our knowledge, possible to solve the classical conflict between the light budget and the sectioning property in three-dimensional microscopy and to provide a virtually unlimited density of foci at best axial resolution. © 2000 Optical Society of America [S0740-3232(00)01906-2]


1. INTRODUCTION

Multifocal multiphoton microscopy (MMM)1 has recently been introduced as a fast and efficient mode of three-dimensional (3D) fluorescence microscopy. In MMM a rapidly scanned array of high-aperture foci nonlinearly excites the fluorescence molecules in the sample.2–5 The strength of MMM is to deliver 3D imaging in real time, directly to the eye if desired, while largely retaining the sectioning capabilities of its nonparallelized, single-beam counterpart.6 Owing to its versatility in life cell imaging, MMM is expected to contribute significantly to the growing popularity of nonlinear imaging modes in microscopy.

Parallelized real-time 3D microscopy has been traditionally accomplished by disk scanning confocal microscopy, with the use of an array of pinholes arranged in a Nipkow-type pattern.7–9 The pinhole array provides both the light for linear excitation and confocal spatial filtering upon detection. The image is formed on a CCD camera or an equivalent detector. Unfortunately, like all parallelized 3D microscopes, this arrangement needs to compromise between, on the one hand, confocality and high axial resolution and, on the other, degree of parallelization and brightness.10,11 The conflict stems from the fact that while reducing the distance between the pinholes increases the light budget, the same measure also leads to a cross-talk between adjacent confocal channels. For example, fluorescence that is generated by the illumination cone of one pinhole may also enter other pinholes.

The cross-talk is best understood by considering that the excitation is performed through an array of illumination point spread functions (I-PSF’s) that coincides with an array of detection PSF’s (D-PSF’s). Whereas the main maximum of the corresponding I-PSF’s and D-PSF’s may be perfectly colocalized in the focal plane, the axially displaced lobes of the I-PSF may overlap with those of the D-PSF of an adjacent pinhole. But, also, the individual I-PSF’s and D-PSF’s can overlap among themselves, so that regions with reinforced excitation or detection are generated. For example, in the case of coherent illumination, at a given axial distance from the focal plane, the sidelobes of the adjacent I-PSF’s may constructively interfere with each other, so that increased excitation and bleaching will occur in the region of constructive interference. As this is a classical problem in parallelized 3D microscopy, considerable research effort has been devoted to it.11

MMM does not require backimaging on a pinhole array because the nonlinear absorption automatically restricts the fluorescence emission to the focal plane. Hence there are no confocal detection channels that could overlap. The overlap of the I-PSF’s may also be present in the MMM but is fortunately ameliorated by the fact that the excitation scales with the cube or the square of the intensity. Nevertheless, its presence has to be taken seriously in a proper system design, especially when the highest possible density of foci is needed, as has also been recognized experimentally.2 A high focal density may become practically relevant when the fluorescence photon flux of a reduced area has to be maximized.

Another innovative, highly parallelized microscope is the programmable array microscope,12,13 which illuminates the sample and detects the image by means of a programmable, movable micromirror device. An elaborate sequence of patterns allows the selection of regions of interest as well as of the degree of confocality. The programmable array microscope is versatile and particularly fast in spectroscopic 3D analysis of a fluorescent sample. However, if used as a highly parallelized confocal system, the cross-talk between the channels has to be considered.

The conflict between light budget and sectioning has also stimulated the introduction of self-correlating aperture microscopy,14 whereby a disk with a very dense and random pattern of pinholes is utilized. Maximizing the density of pinholes maximizes the light throughput and also the cross-talk. In fact, in self-correlating aperture microscopy the cross-talk is fortunately so strong that it
corresponds to an image of a conventional microscope. Scanning with such a disk gives an image consisting of a bright confocal section added to a very bright conventional image. Therefore, after having also recorded a conventional image, one can readily obtain the desired confocal section by subtraction of the images.

Sectioning microscopy by fringe pattern projection is similar. After the acquisition of three images with spatially shifted fringe patterns, one is able to calculate the sectioned image by proper subtraction. While these microscopes excel by their ability to use a conventional light source rather than a (mode-locked) laser, they obtain the sectioned image only after subtraction of two or three images. These images must be taken from the same place under identical conditions. This makes them amenable to noise that is inherently connected with the limited number of fluorescence photons available and image mis-registration. Hence these microscopes will develop their full potential not only, but primarily, with bright, non-bleaching, and non-moving specimens. Owing to its inherent sectioning ability and to the generally lower total photodamage associated with near-infrared (NIR) light, MMM is expected to be superior for fast 3D fluorescence imaging of live cells.

The aim of this paper is twofold. First, we will work out the effective PSF of MMM, with particular emphasis on the relationship between the degree of parallelization and the sectioning strength. In doing so, we provide the theoretical framework for the design of multifocal multiphoton microscopes. Second, we will show that MMM has a unique potential to solve the hitherto unsettled cross-talk resolution conflict. MMM has the potential to allow 3D imaging with a virtually infinite degree of parallelization at an uncompromised axial resolution.

2. MULTIFOCAL MULTIPHOTON MICROSCOPY

Multiphoton excitation microscopy is usually carried out with a mode-locked Ti:sapphire laser providing a train of picosecond or subpicosecond NIR pulses at an 80-MHz rate. The 2–3 NIR photons are absorbed in a single quantum event. The invention of MMM was triggered not only by the general demand for fast high-resolution microscopy but also by the insight that in multiphoton microscopy the signal is normally limited by nonlinear optical processes. As has been demonstrated in the literature, multiphoton excitation microscopy it is usually more important to keep the maximum focal pulse intensity below a certain threshold rather than minimizing the time-averaged power. For 100–200-fs pulses the maximum allowable focal intensity is reportedly approximately 100–300 GW/cm².

When focusing with a high-numerical-aperture (high-NA) lens, this focal peak intensity is already achieved with 5–15 mW of time-averaged power. This amounts to only approximately 1% of the total time-averaged power of a Ti:sapphire laser, so that most of the laser light is usually dumped without being used. In consequence, it is sensible to split up the beam into N beamlets, each sharply focused at the sample, with each beamlet leading to the maximum allowable focal intensity. In consequence, MMM increases the total fluorescence yield by a factor of N. Alternatively, the increase in excitation efficiency can be used for decreasing the image acquisition time by the same factor.

A. Setup

In the initial MMM setup, the laser beam is divided by means of an array of microlenses into typically \( N = 6 \times 6 \) hexagonally arranged beamlets overfilling the back aperture of the objective lens. Thus the beams produce an array of high-resolution I-PSF’s at the focal plane. By positioning the microlenses on a fast rotating spiral, the focal plane is completely raster scanned by the foci. Typically, 375 complete scans per second are achieved, and up to typically 3–4000 frames per second can be anticipated by further increasing the speed of rotation. Alternatively, the array can be scanned by using galvanometric beam deflection. The axial scan is accomplished by moving the sample axially as in standard laser scanning microscopes. The fluorescence is imaged onto a CCD camera as in a conventional epifluorescence microscope. The sensitivity and the readout time of the CCD camera technically determines the speed of imaging. The distance between the foci can be adapted by varying the magnification of the intermediate optics and is typically between 5 and 10 \( \mu m \). As we will show in our calculations, at this distance the effect of the overlap of the I-PSF’s is largely negligible.

![Fig. 1. Principal optical arrangement of a multifocal multiphoton excitation microscope (unfolded). An array of microlenses produces an array of beamlets, in turn producing an array of high-aperture foci in the focal plane inside the specimen. A temporal delay mask (TMX) placed in front of the microlens array ensures that the pulses associated with different foci pass the focal plane at different time points. We refer to this method as time-multiplexed MMM.](image-url)
B. Point-Spread Function

The excitation PSF of MMM for the \(p\)-photon excitation case is described by the \(2p\)-th power (\(p = 1, 2, 3, \ldots\)) of the focal amplitude:

\[
H_{\text{ex}} = [h(x, y, z) \otimes g(x, y, z)]^{2p}.
\]  

(1)

\(h(x, y, z)\) denotes the amplitude PSF of the lens, which is the field generated by a single beamlet:

\[
h(x, y, z) = A \int_0^\alpha \left(\frac{\cos(\theta) \sin(\theta)}{\sqrt{x^2 + y^2}} \right) J_0(k \sqrt{x^2 + y^2} \sin(\theta))
\]

\[
\times \exp[i k z \cos(\theta)] d\theta.
\]  

(2)

\(A\) is proportional to the beam intensity, \(J_0\) is the zero-order Bessel function of the first kind, and \(\theta\) is the polar aperture angle and \(\alpha\) is its maximum. The wave number is given by \(k = 2\pi n/\lambda_0\), where \(\lambda_0\) denotes the vacuum wavelength and \(n\) is the index of refraction of the medium in the focal region. The symbol \(\otimes\) denotes the convolution operation. The function

\[
g(x, y, z) = \sum_{n=1}^N \delta(x - x_n) \delta(y - y_n) \delta(z)
\]  

(3)

is the grating function describing the coordinates of the array that is usually confined to the focal plane. \(\delta\) is Dirac’s delta function. The effective PSF takes into account the lateral scanning procedure and the conventional imaging onto the CCD camera. It is given by

\[
I_{\text{eff}}(x, y, z) = [h(\text{ex}, \text{ey}, \text{ez})]^2 I_s(z),
\]  

(4)

where

\[
I_s(z) = \int \int dx dy [h(x, y, z) \otimes g(x, y, z)]^{2p}
\]  

(5)

denotes the \(z\) response of the microscope and \(\varepsilon = \lambda_{\text{ex}}/\lambda_{\text{em}}\) is the ratio between the excitation and emission wavelengths. The \(z\) response is equivalent to the response of an infinitely thin fluorescence layer when scanned along the optic axis.

The lateral resolution is determined by the first factor in Eq. (4), which is the PSF of a conventional epifluorescence microscope and is given by the lateral extent of the intensity PSF of the lens at the fluorescence wavelength. As this wavelength is shorter than the NIR wavelength, the lateral resolution of MMM is slightly better than that of a standard two-photon beam-scanning system. In the limiting case of a sufficiently sparse grating, say \(x_i - x_{i-1} \gg \lambda_{\text{em}}/\sqrt{N}, \ y_j - y_{j-1} \gg \lambda_{\text{em}}/\sqrt{N},\) the interference effects between the foci are negligible, and Eq. (5) can be rewritten as

\[
I_s(z) = N \int \int dx dy [h(x, y, z)]^{2p} \otimes g(x, y, z)
\]

\[
= N \int dx dy [h(x, y, z)]^{2p}.
\]  

(6)

In this case the multifocal multiphoton microscope has the same \(z\) response as that of a single-beam two-photon excitation microscope, with the only difference being that it is brighter by a factor corresponding to the number of beamlets. If the grating function is dense, the interference of the amplitudes of different foci results in a different \(z\) response and hence in a different axial resolution and sectioning strength. In general, one can expect the axial resolution to be poorer than in the previous case.

Another useful measure of the sectioning strength of a 3D microscope is the sea response

\[
I_{\text{sea}}(z) = \int_{-z}^{\infty} I_s(z') dz'.
\]  

(7)

The sea response describes the signal generated by a fluorescent half-space that is moved along the optic axis. Strictly speaking, the sea response does not give information different from that supplied by the \(z\) response, but it visually emphasizes contributions of weak focal lobes in dense specimens. The behavior of the \(z\) response and the sea response as a function of the density of the grating will be of primary concern in our paper.

C. Parameters of the Calculation

For our calculations we have chosen an objective lens of NA = 1.35 (oil immersion, \(n = 1.518\)), which is typical for high-NA 3D microscopy. We also elected \(p = 2\) and \(3\) to stand for two- and three-photon excitation, respectively. For this set of conditions, the diameter of the Airy

Fig. 2. Arrangement of the foci: (a) conventional hexagonal configuration, (b) three delay subclasses in a regular arrangement, (c) three delay subclasses randomly arranged in a hexagonal pattern.
disk of the I-PSF is approximately $0.8\lambda_0$. As the micro-
lens array is illuminated by the same wave front, it is rea-
sonable to assume that the beamlets are mutually coher-
ent and have the same initial phase. 

Furthermore, we started from a hexagonal arrange-
ment of $N = 37$ high-aperture foci in the focal plane,
which also corresponds to the design of our initial multi-
focal microscope. If the distance between closest neigh-
bors is denoted as $s$, the maximum distance of a focus 
from the center of the array is given by $3s$ [Fig. 2(a)]. 
To evaluate the integrals (5) and (6) efficiently, we had to re-
strict the integration area in a reasonable manner. In 
the case of two-photon excitation ($p = 2$), we have se-
lected the area of integration so that a disk $\approx 2.5$ times 
the area of the radius of the outermost Airy disk was still 
included [Fig. 2(a)]:

$$I_z(z) = \int_{-3s-2\lambda_0}^{3s+2\lambda_0} \int_{-3s-2\lambda_0}^{3s+2\lambda_0} H_{\text{exc}}(x, y, z) \, dx \, dy. \quad (8)$$

We confirmed that when the integration area was en-
larged to $3s + 8\lambda_0$, the upper bound for the relative sys-
tematic deviation was $0.4\%$; usually, it was significantly 
lower. The calculation of the sea response is complicated 
by asymptotic behavior of the $z$ response. When calculat-
ing the integral $I_{\text{sea}}$, one has to delimit the integration 
range along the optic axis. We found that a range of 
$-40\lambda_0 \leq z \leq 40\lambda_0$ was sufficiently large. 
This was sub-
stantiated by the fact that, for interfocal distances larger 
than $\lambda_0$, when the distance was increased to $z_{\text{max}} = 100\lambda_0$, the relative change of the sea response was 
below $1\%$. Hence we calculated that

$$I_{\text{sea}}(z) = \int_{-z}^{40\lambda_0} I_z(z') \, dz'. \quad (9)$$

3. RESULTS

In the following we shall present numerical results of the 
$z$ response and the sea response revealing the relation-
ship between the sectioning strength and the degree of 
parallelization. We will consider both the two- and 
three-photon excitation fluorescence modes, which are in 
practice the relevant excitation modes in microscopy.

A. Response

Figure 3 displays the two-photon excitation $z$ response $I_z$ 
as a function of the interfocal distance $s$ in the range of 
$0 \leq s \leq 13\lambda_0$. For the limiting case of $s \to 0$, the $z$ re-
sponse approaches the unparallelized, single-lens case 
featuring a FWHM of $0.83\lambda_0$, which we shall denote as 
FWHM$_1$. For a spacing narrower than the wavelength 
($0.1\lambda_0 \leq s \leq \lambda_0$), interference between the 3D amplitude 
PSFs broadens the $z$ response and degrades the axial 
resolution. For slightly larger distances, say for $s$
$z \geq 3\lambda_0$, the interference becomes less pronounced, and the axial width of the $z$ response rapidly approaches FWHM$_1$. At a typical wavelength of $\lambda_0 = 800$ nm, the distance of $s = 3.2 \mu m$ leads to a FWHM of $0.78 \lambda_0$, which is close to FWHM$_1$. In Fig. 3(a) we compare the single-beam response $I_z(s = 0)$ with $I_z(s = 5\lambda_0)$ and $I_z(s = 10\lambda_0)$. We find that the two MMM curves differ from the first curve by only a shoulder smaller than 3.5% and 1%, respectively. The calculations reveal that interfocal distances of a few micrometers are sufficient to obtain a high axial resolution in MMM. This is confirmed by the experiment: In the initial setup the interfocal distance for a NA = 1.35 oil lens was set to $s = 5.6 \mu m = 7\lambda_0$.

To provide a better understanding of the physical phenomena, we have highlighted the weak regions by displaying the data of Fig. 3(b) on a semilogarithmic plot [Fig. 3(d)]. We find line-shaped regions of locally increased squared intensity that are parabolically drifting away from the optic axis. These lines are due to the partial constructive interference that typically occurs when periodic structures are illuminated with coherent plane waves. The coherent illumination of a periodic structure, such as a microlens array, results in repeated gradual self-imaging of the foci in characteristic planes perpendicular to the optic axis.\cite{20,21} This is known as the Talbot effect, and the corresponding planes are known as the Talbot and fractional Talbot planes.\cite{22} Whereas in Talbot planes the initial grating pattern is reproduced, in fractional Talbot planes the multiplicity of the pattern is altered. In some planes the pattern may be shifted or even rotated, thus resulting in aesthetically appealing ornaments.

Figure 4 displays the calculated pattern of the squared intensity in the focal plane $z = 0$ and the (fractional) Tal-
bot planes at $3.2\lambda_0$, $5.1\lambda_0$, $10.4\lambda_0$, $16.4\lambda_0$, and $25.7\lambda_0$ for an $s = 5\lambda_0$ grating. For the sake of better visualization, we have normalized the Talbot planes to equal brightness. Fortunately, the squared intensity in the Talbot planes is much weaker than that at the focal plane because the field is blurred with increasing distance $z$ from the focus. This is due to the strong defocusing caused by the large aperture angle of $\sim 120^\circ$. (Strictly speaking, Talbot self-reproduction occurs with finite arrays only when low focusing angles are involved.)

The strength of the squared intensity is shown in the intensity profiles beneath the focal patterns. The highest local squared intensity (11% of the initial value) is found at the “full” Talbot planes, as is the case at $z = 10.4\lambda_0$. In planes with higher multiplicity, such as the one at $z = 3.2\lambda_0$, the squared intensity is lower. Nevertheless, these planes can also contribute to the background because of their multiplicity. In fact, the small peaks found in the semilogarithmically displayed $z$ response [Fig. 3(c)] can be associated with contributions from individual Talbot planes.

B. Fluorescence Sea Response

The $z$ responses in Fig. 3 suggest that for two-photon excitation an interfocal distance of $s > 5\lambda_0$ almost excludes interference effects between the foci. Hence the two-photon-based MMM should display almost the same axial resolution as that of a single-beam two-photon microscope. While this is certainly true for the separation of stacked layers, this may not be true when imaging densely fluorescent media. In this case minute contributions from throughout the sample may add to a significant background that is superimposed on the sectioned image. The strength of this effect can be investigated by tracing the axial response of a fluorescence sea. Therefore the two-photon sea response $I_{sea}(z)$ has been calculated following relation (9) as a function of the interfocal distance $0 \leq s \leq 13\lambda_0$ (Fig. 5).

Figure 5(b) shows $I_{sea}(z)$ as a two-dimensional contour plot featuring the 50% line as the vertical line in the center. The width of the sea response can be inferred from the density of the contour lines. In Fig. 5(a) we compare $I_{sea}(s = 5\lambda_0)$ and $I_{sea}(s = 10\lambda_0)$ with the response $I_{sea}(s = 0)$ found for a single beam. The calculations show that the first two curves differ from the latter by a shoulder of approximately 20% and 12%, respectively. This shoulder is a consequence of the summation of small two-photon excited fluorescence in the uniform sea of fluorophores. Figures 6(a) and 6(b) show, respectively, the FWHM of the $z$ response and the distance between the

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Fig. 5. (a) Normalized two-photon fluorescence sea response $I_{sea}$ for interfocal distances $s = 0\lambda_0$ (curve 1), $s = 5\lambda_0$ (curve 2), and $s = 10\lambda_0$ (curve 3) and (b) contour plot showing $I_{sea}$ as a function of $s$ and the axial coordinate $z$.

Fig. 6. Sectioning in two-photon MMM as a function of the interfocal distance $s$: (a) FWHM of the $z$ response and (b) the $\delta_{20\% - 80\%}$ threshold value of the sea response, normalized to their single-beam counterparts.
20% and 80% values of the sea response, which we denote as $d_{20\% - 80\%}$, as a function of the interfocal distance $s$. It is evident that a fluorescence sea is an artificial object that represents the worst-case situation. Nevertheless, Figs. 5 and 6 indicate that the interfocal distance should ideally not be smaller than $7\lambda_0$ in two-photon imaging of dense fluorescence objects or objects with a vast amount of unbound fluorophore.

It is now interesting to investigate the axial resolution performance of MMM in the three-photon excitation mode. As the fluorescence scales with the cube of the intensity, we can expect that the effect of the various Talbot planes is further reduced. This is also found in the calculations, the results of which are shown in Fig. 7. In fact, Fig. 7 is the three-photon counterpart to the two-photon data of Fig. 5. Whereas Fig. 7(b) shows the three-photon MMM sea response as a contour plot, Fig. 7(b) compares $I_{\text{sea}}(s = 2.5\lambda_0)$ and $I_{\text{sea}}(s = 5\lambda_0)$ with the single-beam response $I_{\text{sea}}(s = 0)$. The comparison reveals that for interfocal distance large than $3\lambda_0$, that is, 2.5–3 μm, three-photon MMM can be carried out without compromising the axial resolution.

C. Time-Multiplexed Multifocal Multiphoton Microscopy

The results presented in Subsections 3.A and 3.B disclose that for two- and three-photon MMM of high NA, the interfocal distance $s$ should preferably not be smaller than $7\lambda_0$ and $3\lambda_0$, respectively. For most applications this distance or slightly larger distances are just adequate. In fact, moving the foci closer is usually not desired because of the concomitant decrease in the field of view. For example, with a typical array of $6 \times 6$ foci, the interfocal distance of $s = 7\lambda_0$ is accompanied with a field of view of 42 μm in diameter. Nevertheless, it might be desirable to decrease the interfocal distance because a higher density of foci would result in a higher total excitation rate. Hence the total fluorescent flux from the illuminated region would be increased. This measure may even become necessary when a particular region has to be imaged at high speed.

To maintain the axial resolution at small interfocal distances, we have to prevent interference from taking place. As MMM relies on excitation with laser pulses of ultrashort (picosecond or subpicosecond) duration that are approximately 13 ns apart, we can define a straightforward way to avoid focal overlap. As has also been independently recognized by Buist et al., the introduction of a temporal delay between the beamlets ensures that light pulses of neighboring foci pass the focal region at different time points. Interference will not occur because the beamlets simply do not meet each other. Of course, this measure has no influence on the total illumination efficiency because multiplexing the arrival times of the pulses at the sample has no influence on the total number of excitation pulses.

The temporal delay is chosen such that it is slightly larger than the pulse duration. For 100-fs pulses, temporal delays of $t_i \geq 2t_i \approx 200$ fs are already sufficient for the desired time-multiplexing effect. A possible method to realize such an optical delay is to place cylinders of transparent optical material of varying thickness $d_i$ in front of the lenses. In the case of a glass cylinder with the refractive index $n$, a temporal delay $t_i$ is achieved by a glass thickness of $d_i = t_i c/(n - 1)$ with respect to a non-delayed pulse. The constant $c$ denotes the speed of light in air, and the index $i = 0, 1, \ldots, i_{\text{max}} = 1/(2f r) \approx 62,500$. Hence a delay of $i \times 100$ fs is achieved by a glass thickness of $d_i = i \times 60$ μm. The large number of potential time delays, $i_{\text{max}}$, is a direct consequence of the disparity between the pulse duration and the time gap $1/f$ between the pulses. For practical reasons one is probably limited to a maximum glass thickness of a few millimeters, so that $i_{\text{max}} = 100$ might be a more realistic value. Nevertheless, as $N \ll i_{\text{max}}$, we would be able to time-multiplex virtually all the lenses so that only one pulse resides in the sample at a given time point. We refer to this microscopy method as time-multiplexed MMM.

In most cases it will be sufficient to time-multiplex neighboring pulses that would otherwise overlap in the focus. Hence one has to balance the grating distance $s$ with respect to the number $i$ of time-multiplexed foci. It
is therefore interesting to investigate the effect of a finite number \( i \) on the axial resolution, in particular in borderline cases, e.g., with three different pulse arrival times \( (i = 2) \). Figure 8 compares the sea response \( I_{\text{sea}} \) for (a) \( s = 5\lambda_0 \), regular (curves a); \( s = 5\lambda_0 \), with an optical delay as in Fig. 2(b) and three regular subclasses (curves b); \( s = 8.6\lambda_0 \), regular arrangement (curves c).

The periodic arrangement of an optical delay mask with a finite delay \( (i = N) \), as shown in Fig. 2(b), still produces Talbot planes, although of considerably reduced squared intensity. Therefore one is tempted to ask whether it would be more helpful to arrange the delay mask in random order, as sketched in Fig. 2(c). Figures 9(a) and 9(b) show an enlargement of the part sensitive to the interfocal distance of the two-photon excitation fluorescence sea and \( z \) response, respectively. Figure 9 shows them for the cases without TMX (curves a), with regular TMX, as in Fig. 2(b) and (curves b), and for random TMX, arranged in three different patterns that are denoted as curves r2, r3, and r4, referring to two, three, and four different phase delays \( (i = 1, 2, 3) \), respectively. The computations confirms that the cross-talk decreases rapidly with increasing \( i \). In addition, increasing the multiplicity by 1\((i = i + 1)\) is more effective for small values of \( i \) than for larger values, as expected. We also find that for an equal number of foci in the same subgroup, a random pattern is not very different from its periodic counterpart. However, the random arrangement effectively prevents the formation of well-defined Talbot planes, so that the responses show a largely monotonic decline in fluorescence intensity.

4. DISCUSSION, OUTLOOK, AND CONCLUSION

We have investigated the axial resolution of a multiphoton multifocal microscope as a function of the degree of parallelization. Our study is highly relevant to its optical design, in particular with respect to the right choice of the distance between individual foci in the focal plane. For two-photon excitation at high-aperture angles (NA = 1.35 oil, \( \alpha = 63^\circ \)), we could establish a minimum interfocal distance of \( s \geq 7\lambda_0 \). Hence, in properly de-
signed MMM, the interfocal distance should be approximately 5–6 μm. For smaller interfocal distances interference between individual foci occurs in the region a few micrometers above and below the focal plane, resulting in a gradually decreased sectioning strength. If the images are bright enough, this can be adequately compensated by subtraction of a background. For large interfocal distances the axial resolution is largely that of a single-beam two-photon excitation microscope, whose axial resolution is given by the squared intensity PSF of the objective lens.

In the case of three-photon excitation, the cubic nonlinearity leads to a stronger suppression of the weak interference contributions. Lower interfocal distances $s \geq 3\lambda_0$, typically 2.4 μm, are sufficient for obtaining a sectioning strength similar to that of a single beam. Thus three-photon and higher orders of excitation allow for an even higher degree of parallelization as compared with that from the two-photon case. The cubic order of excitation requires careful adjustment of the beam intensity to the upper allowable intensity values, which are of the order of 100–300 GW/cm². This is required because low intensity levels yield poor signals. In conclusion, MMM provides a good solution for this problem because it allows the dense distribution of multiple foci with safe intensity levels, thus leading to a multiple increase in brightness.

Although MMM does not require confocal pinholes for sectioning, it can be combined with a confocal pinhole disk. Confocalization adds complexity to the microscope, such as the requirement to align the microlens array and the pinhole array and also the necessity for compensating for the longitudinal chromatic aberration between the NIR light and the fluorescence. Another technical challenge is the requirement to place the dichroic mirror between the pinhole array and the microlenses, unless a spatially remote but perfectly synchronized pinhole disk is used. In mathematical terms, however, confocalization means that the squared or cubic illumination intensity PSF array would be multiplied by an array of detection PSFs calculated at the emission wavelength. This would result in an additional suppression of out-of-focus signal and therefore to a further improvement of the axial resolution. Another effect is that the foci can be moved significantly closer than the previous $3\lambda_0$ or $7\lambda_0$ simply because the multiplication with a detection PSF is, in first approximation, similar to an increase of the order of excitation.

We have shown that a high degree of parallelization is accomplished by time-multiplexing the pulses through an optical delay mask. Although our initial calculations assumed a pulse length of $\tau = 100$ fs, that is, the pulse length of the laser system now available to us, focal pulse lengths on the order of 10 fs have been demonstrated.23 With such short pulses a glass thickness of $d_i = i \times 6$ μm is sufficient for an effective delay. Significantly, it would technically allow us a total number of $i_{\text{max}} = 1000$ time-delayed illumination channels. We note that the interfocal cross-talk can be reduced to a lesser extent by phase multiplexing of the different beamlets or by the use of different polarization states.

The concept of time-multiplexed pulse excitation opens up the prospect of very fast 3D imaging at the highest possible resolution. In fact, any confocal microscope using pulses for excitation can be time multiplexed, irrespective of whether it relies on first, second, third, or higher order of excitation. For example, time-multiplexed multifocal single-photon confocal microscopy will eliminate the interference between the individual excitation foci. With the decreasing costs of pulsed laser systems, time-multiplexing confocal microscopes might be attractive in the future. TMX of the nanosecond duration fluorescence pulse is conceivable but is technically complex because it may require a time-gated detector with a fast readout. However, TMX of coherent nonlinear processes, such as multifocal second and third harmonics as well as coherent anti-Stokes Raman scattering imaging, will be attractive because it would significantly reduce the coherent cross-talk between the signal beams.

Significantly, the concept of time-multiplexed MMM solves the classical conflict between brightness and sectioning strength and should for the first time allow 3D imaging at a virtually unlimited degree of parallelization. However, the most fascinating option is to position the time-multiplexed foci so close that one does not require scanning. In that case the whole-field sectioned image is obtained just by illumination.

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