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Is phase-mask alignment aberrating your STED microscope?

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Abstract

The performance of a stimulated emission depletion (STED) microscope depends critically upon the pupil plane phase mask that is used to shape the depletion focus. Misalignments of the mask create unwanted distortions of the depletion focus to the detriment of image quality. It is shown how the phase errors introduced by a misplaced mask are similar to coma aberrations. The effects are investigated analytically, through numerical modelling and experimental measurement. Strategies for the systematic alignment of the masks are discussed.

1. Introduction

Recent developments in fluorescence nanoscopy are creating wide ranging benefits in biomedical imaging by providing enhanced resolution of sub-cellular structures. Stimulated emission depletion (STED) microscopy is one such method, which provides super-resolution by restricting the region of fluorescence emission to a size far smaller than the diffraction limited focus of the excitation laser beam \cite{1,2}. Super-resolution is achieved by superimposing the excitation focus with the focus of another laser, which is structured to have a region of minimum (and ideally zero) intensity at the centre of a bright ring. The high-intensity second focus—known as the depletion focus—is used to force excited molecules to undergo stimulated emission and in so doing prevent spontaneous fluorescence emission. The aim of the stimulated emission process is to ensure that the remaining excited molecules are confined to a small region at the centre of the focus, near to the zero intensity point of the depletion beam. As the stimulated emission depletion effect can be saturated, it is possible to reduce the size of the effective fluorescence region by increasing the intensity of the depletion beam. In practice this has lead to resolutions of 20 nm in living cells \cite{3} and 2.4 nm when imaging defects in diamond \cite{4}.

The performance of a STED microscope depends critically on the quality of the depletion focus and in particular on the nature of the minimum intensity point. For highest resolution operation, the minimum intensity should be as close to zero as possible and the shape of the surrounding high-intensity ring should be uniform. As a non-zero minimum intensity will deplete the fluorescence, a poor quality minimum intensity cannot be effectively compensated by increasing the collection time or excitation laser intensity. Conventionally, the focal shaping is implemented by imaging a phase mask onto the objective lens pupil. This mask is typically a shaped transparent plate that imposes an appropriate phase pattern on to the incoming laser beam. Other implementations have instead used a liquid crystal spatial light modulator (SLM) to introduce the required phase pattern into the depletion beam \cite{5–7}. This approach is more versatile, as it provides additional flexibility in defining the properties of the phase pattern. An advantage of the SLM as a phase mask generator is that any required mask can be corrected for changes in the depletion wavelength—conventional phase masks are manufactured for a fixed wavelength and if the laser differs from the design wavelength or there are manufacturing defects then the mask will perform sub-optimally. The quality of the zero-intensity region generated by SLMs is therefore comparable in the optimum case to that generated by a conventional phase mask. We believe that, due to the dynamic configuration flexibility offered by the SLM, in practical implementations this flexibility will lead to superior performance for SLM-based STED microscopes. An important consideration, however, is that the SLM must be correctly imaged onto the back aperture of the microscope objective; there is not the same tolerance of axial positioning errors observed with the conventional phase mask. In either implementation, the lateral position of the phase mask affects the form of the depletion focus and correct positioning is essential.
in obtaining the optimum performance from the STED microscope. In conventional STED systems, the phase mask is adjusted using lateral translation stages in an iterative procedure; in the SLM-based systems the translation is implemented by changing the displayed phase pattern, which also facilitates automated alignment [8]. Alignment can be performed by inspection of the depletion focus shape by scanning a small gold bead near the focus and through detection of the scattered light. Incorrect placement of the phase mask results in distortion of the depletion focus, which can be detected visually by asymmetry in the acquired images. Another method has used phase retrieval from focal images to obtain estimates of the pupil plane phase [9]. Irrespective of the method used to impart the phase pattern on the beam, it is necessary to ensure a well-defined polarisation in order to correctly generate the depletion focus [10, 11].

The quality of the depletion focus is also affected by aberrations—phase distortions in the optical wave fronts—which can be inherent in the optical system or induced by spatial variations in refractive index within the specimen [12, 13]. The aberrations distort the focal intensity distribution leading to a drop in achievable resolution and, in some cases, cause an increase in the minimum intensity at the centre of the focus, which additionally causes loss of detected fluorescence [14]. The recent introduction of adaptive optics to STED microscopy has enabled correction of specimen induced aberrations and improved super resolution imaging through thick tissue [7].

In this paper, we investigate the link between phase mask misalignment and aberrations. Wave front aberrations can be thought of as an error between the desired, ideal wave front phase (such as that of a spherical wave converging to the focus) and the phase of a wave front distorted by propagation through refractive index structure of the specimen. A misplacement of the phase mask in effect also introduces a phase error, as the actual induced phase function will be different to the desired function for creation of the shaped depletion focus. This phase error is analogous to an aberration of the depletion beam. We present theoretical analysis that shows the correspondence between phase mask position error and aberration for the most common two-dimensional (2D) and three-dimensional (3D) STED configurations. In particular, it is shown that the predominant aberration mode linked to phase mask misalignment is coma. Furthermore, the similarity between these effects could lead to the inadvertent compensation of coma present in the system by phase mask displacement when aligning conventional STED microscopes. The misalignment can only partially compensate for coma and so will leave the system running sub-optimally. The analysis of the effects of phase-mask misalignment is compared to both numerical modeling and experimental measurements. An improved understanding of these effects will be useful in the design of automated alignment systems that are also robust to the effects of aberrations.

2. Analysis of phase mask misalignment

The most common implementation of STED microscopy—referred to here as the 2D STED mode—uses a helicoidal phase mask to create a depletion focus with a zero intensity along the entirety of the optical axis. The effect of this phase mask is to provide 2D confinement of fluorescence, creating resolution enhancement in the lateral (\(xy\)) plane, but not enhancement along the direction of the optical axis (z direction). 2D STED is demonstrated in figure 1, where the \(zx\) plane of the structured focal volume is shown in (b), the resulting fluorescence emission in (c) and the phase mask used to generate the STED beam in (h). The alternative 3D STED mode uses a \(\pi\)-step phase mask, consisting of concentric circular and annular regions with a relative phase shift between them of \(\pi\) radians. This phase mask creates a region of minimum (ideally zero) intensity surrounded in all directions by non-zero intensity. The resulting resolution enhancement occurs in three dimensions as shown in figures 1(e), (f) and (i). The effects of phase mask misalignment on the 2D and 3D STED modes are considered separately in the next sections.

2.1. 2D STED mode

For 2D STED, the helicoidal phase mask can be represented by the complex transmission function

\[
T_{2D}(r, \phi) = e^{i\phi}
\]

where \((r, \phi)\) are the polar coordinates that describe the pupil plane of the system, which is assumed to have unit radius. Using the geometry illustrated in figure 1(g), where the centre of the phase mask has been shifted from the origin O to a point \(C\), offset a distance \(x_0\) along the x axis, we can write the transmission of the misaligned phase mask as

\[
T'_{2D}(r, \phi) = e^{i\phi} e^{i(\phi' - \phi)}
\]

(2)

The misalignment of the phase mask thus manifests itself as a phase error (or aberration) given by the function \(\psi(r, \phi) = \phi' - \phi\). It is convenient at this stage to note from the geometry that

\[
\tan \phi' = \frac{r \sin \phi}{r \cos \phi - x_0}
\]

(3)

We now use the trigonometric identity

\[
\arctan u - \arctan v = \arctan \left( \frac{u - v}{1 + uv} \right)
\]

(4)

with \(u = \tan \phi'\) and \(v = \tan \phi\) to derive the phase aberration as

\[
\psi(r, \phi) = \phi' - \phi = \arctan \left( \frac{x_0 \sin \phi}{r - x_0 \cos \phi} \right)
\]

(5)

Using the approximation \(r \gg |x_0|\), which is valid across all but the central region of the pupil for small misalignments, then the aberration simplifies to
In the region near the origin, where \( r \ll |x_0| \), we find that \( \psi(r, \phi) = -\phi \), which in effect cancels out the original phase mask giving \( T'_{3D}(r, \phi) = 1 \). The form of the right hand side of equation (6) infers that a small offset of the phase mask in the \( x \) direction has a similar effect to the introduction of a coma-like aberration, whose direction of maximum variation is oriented along the \( y \) axis. Extension of this result to other misalignment directions is simply achieved by a rotation of the coordinate system about the optic axis.

**2.2. 3D STED mode**

For 3D STED, the pi-step phase mask can be represented by the complex transmission function

\[
T_{3D}(r, \phi) = e^{i\xi(r, \phi)}
\]

whose phase \( \xi \) is given by

\[
\xi(r, \phi) = \begin{cases} 
0 & 0 \leq r < \beta \\
\epsilon - \pi & \beta \leq r \leq 1
\end{cases}
\]

where \( \beta \approx 1 / \sqrt{2} \) is chosen to ensure destructive interference at the centre of the focus and \( \epsilon \) is a constant, chosen to ensure for mathematical convenience that the mean value of \( \xi \) is zero. This phase function can be represented by a Fourier series, which it is useful to express as a function of \( r^2 \):

\[
\xi(r, \phi) = \sum_{n=1}^{\infty} A_n \cos (n\pi r^2)
\]

The phase introduced by an equivalent offset phase mask is given by \( \xi'(r, \phi) = \xi(r', \phi') \), where \( r' \) and \( \phi' \) are shown in figure 1(g). Geometric considerations show that

\[
r'^2 = x_0^2 + r^2 - 2x_0 r \cos \phi \approx r^2 - 2x_0 r \cos \phi
\]

where the approximation holds for \( r \gg |x_0| \). Combining this with equation (9) we obtain

\[
\xi'(r, \phi) = \sum_{n=1}^{\infty} A_n \cos \left[ n\pi (r^2 - 2x_0 r \cos \phi) \right]
\]

As outlined in the appendix A, equation (11) can be expanded as a series of separate azimuthal orders in \( \phi \) to give an expression in the form

\[
\xi'(r, \phi) = f_0(r) + \sum_{k=0}^{\infty} f_{k}(r) \cos [(2k + 1)\phi] + \sum_{l=1}^{\infty} g_l(r) \cos (2l\phi)
\]

which for small mask displacements is dominated by the lowest order \( f_0(r) \) and \( f_1(r) \) terms:

\[
\xi'(r, \phi) \approx f_0(r) + f_1(r) \cos (\phi)
\]

The first term \( f_0(r) \) is equivalent to the correctly positioned phase mask \( \xi(r, \phi) \). The second term has the mathematical form of a coma aberration oriented...
along the x-axis. It follows that a small offset of the phase mask in the x direction is similar to the introduction of an x-oriented coma aberration.

3. Demonstration of correspondence between mask misalignment and coma

In order to compare the effects of phase mask misalignment and coma aberrations, we will show results from both numerical modelling and from experimental measurement. The focal field of the depletion beam can be calculated using vectorial theory as

\[
E(x, y, z) = \frac{E_x}{E_z} = \frac{\lambda f}{2\pi \lambda} \int_0^{2\pi} \int_0^{\alpha} E_0(\theta, \phi) T(\theta, \phi) \sin \theta \sin \phi \, \theta \, d\theta \, d\phi
\]

where \( f \) is the focal length of the objective, \( \lambda \) is the wavelength, \( E_0(\theta, \phi) \) is the illumination profile from the laser, \( T(\theta, \phi) \) is the phase mask for generation of the zero intensity focus, \( S(\theta, \phi) \) models the aberrations in the pupil. The \( \sqrt{\cos \theta} \) is the apodisation term for an objective lens obeying the sine condition. The propagation term \( \zeta(\theta, \phi; x, y, z) \) is defined as

\[
\zeta(\theta, \phi; x, y, z) = \exp \left[ ik(x \sin \theta \cos \phi + y \sin \theta \sin \phi + z \cos \theta) \right]
\]

where \( k = 2\pi n/\lambda \). The integration takes place over a spherical cap described by the polar coordinates \((\theta, \phi)\), where the upper limit on \( \theta = \alpha \) is equal to the semi-aperture angle of the objective lens. The focal intensity is calculated as

\[
I = |E|^2 = |E_x|^2 + |E_z|^2 + |E_z|^2
\]

We modelled the depletion focus in Matlab for a range of applied aberrations and focal shifts, using both the 2D and 3D phase masks. In order to observe the effect of coma on the depletion focus, we applied the 7th Zernike polynomial for coma aligned to the x-axis

\[
Z_7(r, \phi) = \sqrt{8} (3r^3 - 2r) \cos \phi
\]

and the 8th Zernike polynomial for coma aligned to the y-axis

\[
Z_8(r, \phi) = \sqrt{8} (3r^3 - 2r) \sin \phi
\]

where \( \sqrt{8} \) is a normalisation factor chosen so that the root mean square phase value of a unit mode is one radian.

Experimental measurements were performed using a custom built adaptive STED microscope incorporating a liquid crystal SLM for implementation of the phase masks and to introduce aberrations. Figure 2 shows a schematic of the system. The STED pulses were supplied by a titanium sapphire laser (Spectra Physics Tsunami) providing 100 fs pulses at a repetition rate of 80 MHz and a centre wavelength of 780 nm. The pulses were stretched to a duration of a few hundred ps by passing them through a 180 mm prism of SF6 glass and then 100 m of polarisation maintaining single mode fibre. At the fibre output the pulse train was collimated and reflected off a liquid crystal SLM (Hamamatsu LCOS), which was used to generate the phase masks required for STED imaging. We also used the SLM to generate Zernike polynomials for correction of aberrations specific to the STED beam. The STED beam was combined with the fluorescence excitation and detection paths at a dichroic filter and was then reflected off a deformable mirror (DM) which allowed for aberration correction of all three microscope beam paths (excitation, emission, depletion). For the purposes of the results presented here, this DM was set to a constant shape that provided diffraction-limited imaging of the single sample used for all images in this paper. The beam was scanned in the xy plane of the sample using a pair of galvonometers mirrors and z positioning was provided by an objective lens piezo-mount (Physik Instrumente Pifoc P-725). In order to ensure the correct circular polarisation of the depletion beam, we incorporated \( \lambda/2 \) and \( \lambda/4 \) waveplates in the setup and used a polarisation analyser (Schaeffer + Kirchhoff SK010PA-VIS) to confirm the polarisation state. The microscopic objective was a 1.4 NA 100 x oil immersion objective (Olympus UPLSAPO 100XO). Two detection paths were available. The first allowed confocal and STED imaging through an emission filter centred at 690 nm with a 50 nm bandpass. A 50 \( \mu m \) core diameter fibre, corresponding to 1 Airy unit, was used as the detection pinhole and an avalanche photodetector collected the signal. The other detection arm used wide area detection to allow measurement of both the excitation and STED foci. This path comprised a pellicle in the descanned beam path that picked off approximately 5% of the reflected light from the sample. A 40 \( \mu m \) diameter region of the sample was then imaged onto a 500 \( \mu m \) core optical fibre that was coupled to another avalanche photodetector. The sample under investigation comprised a mix of 100 nm crimson beads (Invitrogen) used for confocal and STED fluorescence imaging and 150 nm gold beads used for imaging of the excitation and STED foci by backscattered detection in the wide area detector path. The beads were drop cast onto the surface of a standard #1 coverslip which was then mounted on a microscope slide using immersion oil as the mounting medium.

Figure 3 shows the effect of the phase mask misalignment on the STED beam foci for both 2D and 3D
The applied misalignment was 13% of the objective pupil radius. Slices in the $xy$, $xz$ and $yz$ planes, along with the applied misaligned phase-mask are shown. The next sections of this paper investigate the phenomena in more detail.

3.1. 2D STED mode

We first examined the comparative effects of phase mask misalignment and coma on the 2D STED depletion focus. Figure 4 shows how phase mask misalignment errors of up to 13% (equivalent to a lateral positioning...
offset of 1.2 mm on our 9.3 mm diameter phase mask) distorted the focus. Small amounts of misalignment are best noticed by their effect on the central zero intensity line, which tilts from the z axis. With increasing misalignment, the lobes either side of the zero also shift in the z direction relative to one another. For a fixed xy slice, this shift would appear as an asymmetry in the brightness of the ring.

Figure 5 shows the effect of coma, as represented by Zernike mode 8, on the depletion focus. Up to an applied amplitude of 0.5 radians, the dominant effect was to increase the lateral asymmetry of the focus. This distortion was accompanied by a lateral shift of the zero intensity position. With increasing aberration, the central zero became distorted and secondary low intensity regions appeared.

There are some differences apparent between the two examples, as we have used only the lowest order, Z8, coma mode over an arbitrarily chosen range, so one should not expect an exact correspondence between the two sets of data. However, the similarities that do exist between the distortions caused by coma and mask misalignment mean that distinguishing between them in experimental data can be difficult. For example, we had intentionally chosen here the displacement direction, the orientation of the coma mode and observation plane to best illustrate the effects. In practice, the correct orientations would not be known and a section in an arbitrary direction would not necessarily show the difference. In practical terms, this makes it difficult to distinguish between small amounts of coma and mask misalignment. The conventional technique of minimising the tilt in xz and yz of the central zero is not therefore recommended to be used as a primary alignment procedure when a 3D mask is also available in the system (see below).

3.2. 3D STED mode
Using the 3D STED phase mask, we applied equal amounts of phase mask misalignment and coma as were applied for the 2D phase mask, with the appropriate change to Zernike mode Z7 to ensure the correct orientation. Figure 6 shows that, over the applied misalignment range, the depletion focus exhibits little lateral distortion of the focal plane intensity toroid. Rather, the dominant manifestation of the mask misalignment is a tilt of the axis through the centroids of the lobes of the focal distribution above and below the focal plane. This characteristic signature of mask misalignment in an arbitrary orientation could be detected by the acquisition of, for example, orthogonal xz and yz images.

In contrast, figure 7 shows that the dominant effect of coma (Z7) is upon the ring pattern in the focal plane. The intensity profile becomes asymmetrical with increasing coma, but is readily apparent with even small amounts. While there is also an effect on the upper and lower lobes of the PSF, it comes in the form of a curvature that does not induce tilt in the position of the centroids relative to one another. Therefore it is still
Figure 5. Effects of the Zernike coma aberration mode $Z_8$ on the 2D STED depletion focus, as the mode coefficient was varied from $-1$ radian at the left to $1$ radian at the right in steps of $0.5$ radians. Top: numerical modelling. Bottom: experimental measurement.

Figure 6. Effects of phase mask shift on the 3D STED depletion focus, as the position was varied from $x_0 = -0.13$ at the left to $x_0 = 0.13$ at the right in steps of $\delta x_0 = 0.065$. Top: numerical modelling. Bottom: experimental measurement.
possible to distinguish coma from phase mask misalignment for this type of PSF.

The use of the SLM means that it is straightforward to switch between 2D and 3D STED modes without the relative misalignment that might occur when phase masks are physically exchanged. As the effects are less ambiguous for the the 3D STED mask, it would therefore be possible to use the 3D mode to check mask alignment, before switching back to the 2D mode and we would strongly recommend performing phase mask alignment with the 3D mask whenever available.

4. Discussion and conclusion

We have shown through analysis, numerical modelling and experimental measurements for both 2D and 3D STED microscopes that phase mask misalignment and coma aberrations can create similar distortions of the depletion focus. These distortions change the shape of the minimum intensity region and the surrounding intensity field, which affects the size and shape of the remaining non-depleted fluorescence, leading to a detrimental effect on the resolution of the microscope.

Through examination of xz scans of gold beads in the depletion focus, it is possible to discern the differences in distortions caused by coma and mask misalignment. These differences are much more difficult to detect purely by using xy sections of the depletion focus. Interpretation of the corresponding effects on STED fluorescence images is even more challenging. If coma is present in the microscope, either through system or specimen-induced aberrations, there exists the risk of misinterpretation of the effects as being due to mask alignment errors. If the coma is static systematic error, then one could possibly use mask re-alignment as a method of coma compensation, although the correction would only be approximate as the corresponding phase functions would not perfectly cancel out. Specimen-induced coma is likely to vary between specimens, so mask translation would not be a practical solution. Furthermore, applying mask translation as a method of correcting coma to successive samples runs the risk of iteratively walking the system out of alignment due to the commensurate overlap realignment of the excitation and STED beams required when imperfectly correcting for aberrations.

A further complication could occur in systems that permit switching between 2D and 3D STED modes, such as those using a SLM for phase mask implementation. Using mask translation only to compensate, for example, x-oriented coma would require the translation of the phase mask in the x direction for 3D STED, but in the y direction for 2D STED. Adjustment of the mask position for optimum 3D imaging would mean the mask is in a non-optimum position for the 2D STED mode. Furthermore, sequential re-optimisation whilst switching between imaging modes could lead to compounded alignment errors. It is therefore essential that
the effects of coma and mask alignment can be separated through appropriate mechanisms, for example using adaptive feedback control. We suggest that the 3D phase mask is particularly suited to such aberration discrimination and should be used when available, even if the microscope is primarily used to image in the 2D mode.

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Appendix A

In this appendix A we present details of the expansion of the phase error induced for a misalignment of the 3D STED phase mask. Starting from equation (11), we can expand the cosine terms in the Fourier series as follows:

\[ \xi^\prime(r, \phi) = \sum_{n=1}^{\infty} A_n \cos[n \pi (r^2 - 2x_0 \phi \cos \phi)] \]

\[ = \sum_{n=1}^{\infty} A_n \{ \cos(n \pi r^2) \cos(2n \pi x_0 \phi \cos \phi) + \sin(n \pi r^2) \sin(2n \pi x_0 \phi \cos \phi) \} \]

Defining an alternative azimuthal angle \( \chi = \pi/2 - \phi \) allows us to write this as

\[ \xi^\prime(r, \phi) = \sum_{n=1}^{\infty} A_n \{ \cos(n \pi r^2) \cos(2n \pi x_0 \phi \sin \chi) + \sin(n \pi r^2) \sin(2n \pi x_0 \phi \sin \chi) \} \]

which then permits use of the following identities [15]

\[ \cos(x \sin \theta) = J_0(x) + 2 \sum_{k=1}^{\infty} J_{2k}(x) \cos(2k \theta) \]

\[ \sin(x \sin \theta) = 2 \sum_{k=0}^{\infty} J_{2k+1}(x) \sin[(2k+1) \theta] \]

where \( J_n(\cdot) \) represents the \( n \)th order Bessel function of the first kind. Equation (A.2) can then be expressed as a Fourier series in \( \chi \):

\[ \xi^\prime(r, \phi) = F_0 + \sum_{k=0}^{\infty} F_k \sin[(2k + 1)\chi] \]

\[ + \sum_{l=1}^{\infty} G_l \cos(2l\chi) \]

where the coefficients are given by

\[ F_0 = \sum_{n=1}^{\infty} A_n \cos(n \pi r^2) J_0(2n \pi x_0 \phi) \]

\[ F_k = 2 \sum_{n=1}^{\infty} A_n \sin(n \pi r^2) J_{2k+1}(2n \pi x_0 \phi) \]

\[ G_l = 2 \sum_{n=1}^{\infty} A_n \cos(n \pi r^2) J_{2l}(2n \pi x_0 \phi) \]

The relative magnitude of these coefficients can be estimated as the value the summations over \( n \) is bounded by the corresponding continuous integrals in \( n \). If the radial position of the step in the phase mask is set at \( \beta = 1 / \sqrt{2} \) then the Fourier series of equation (9) represents a square wave and the coefficients \( A_n = 1/n \) for odd values of \( n \) and zero for even values of \( n \). We can then make use of the following integrals (from equations (11.4.35) and (11.4.36) in [15])

\[ \int_0^{\infty} \frac{1}{n} J_n(an) \sin(bn) \, dn = \frac{a^n \sin\left(\frac{\pi}{2} \mu \right)}{\mu \left[b + (b^2 - a^2)^{1/2}\right]} \]

\[ \int_0^{\infty} \frac{1}{n} J_n(an) \cos(bn) \, dn = \frac{a^n \cos\left(\frac{\pi}{2} \mu \right)}{\mu \left[b + (b^2 - a^2)^{1/2}\right]} \]

which are valid for \( b \gg a > 0 \) and where we substitute \( a = 2 \pi x_0 \phi \) and \( b = \pi r^2 \). When \( |x_0| \ll r \), which is the case over most of the pupil, the denominators can be approximated by \( \mu (2b)^n \). Following substitution of \( \mu = 2k + 1 \) corresponding to equation (A.7) we therefore obtain

\[ \int_0^{\infty} \frac{1}{n} J_{2k+1}(an) \sin(bn) \, dn \approx \frac{a^{2k+1}(-1)^k}{(2k+1)(2b)^{2k+1}} \]

\[ \sim \left(\frac{x_0}{r}\right)^{2k+1} \]

and substitution of \( \mu = 2l \) corresponding to equation (A.8) leads to

\[ \int_0^{\infty} \frac{1}{n} J_{2l}(an) \cos(bn) \, dn \approx \frac{a^{2l}(-1)^l}{2l(2b)^{2l}} \sim \left(\frac{x_0}{r}\right)^{2l} \]

We therefore conclude that

\[ F_k \sim \left(\frac{x_0}{r}\right)^{2k+1} \]

\[ G_l \sim \left(\frac{x_0}{r}\right)^{2l} \]

which means that equation (A.5) is dominated by the lowest azimuthal order terms. The lowest order term \( k = 0 \) corresponds to the \( \sin \chi \) term—or equivalently the \( \cos \phi \) term—which represents comatic aberration modes. We conclude that coma is the dominant aberration arising from the misplaced phase mask. Also note that the functions \( f_k \) and \( g_l \) used in the main text are simply obtained from the expressions for \( F_k \) and \( G_l \) by expressing \( \xi^\prime \) in terms of the angle \( \phi \) rather than \( \chi \).
References

[1] Hell S W and Wichmann J 1994 Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy Opt. Lett. 19 780

[2] Maglione M and Sigrist S J 2013 Seeing the forest tree by tree: super-resolution light microscopy meets the neurosciences Nat. Neurosci. 16 790–7

[3] Goettfert F, Wurm C A, Mueller V, Berning S, Cordes V C, Honigmann A and Hell S W 2013 Coaligned dual-channel sted nanoscopy and molecular diffusion analysis at 20 nm resolution Biophys. J. 105 L01 –3

[4] Wildanger D et al 2012 Solid immersion facilitates fluorescence microscopy with nanometer resolution and sub-ngstrom emitter localization Adv. Mater. 24 309–13

[5] Donnert G, Keller J, Medda R, Andrei M A, Rizzoli S O, Ihrmann R, Jahn R, Eggeling C and Hell S W 2006 Macromolecular-scale resolution in biological fluorescence microscopy Proc. Natl Acad. Sci. 103 11440–5

[6] Auksorius E, Boruah B R, Dunsby C, Lanigan P M P, Kennedy G, Neil M A A and French P M W 2008 Stimulated emission depletion microscopy with a supercontinuum source and fluorescence lifetime imaging Opt. Lett. 33 113–5

[7] Gould T J, Burke D, Bewersdorf J and Booth M J 2012 Adaptive optics enables 3d STED microscopy in aberrating specimens Opt. Express 20 20998–1009

[8] Gould T J, Kromann E B, Burke D, Booth M J and Bewersdorf J 2013 Auto-aligning stimulated emission depletion microscope using adaptive optics Opt. Lett. 38 1860–2

[9] Kromann E B, Gould T J, Juette M F, Wilhelm J E and Bewersdorf J 2012 Quantitative pupil analysis in stimulated emission depletion microscopy using phase retrieval Opt. Lett. 37 1805–7

[10] Hao X, Wu T and Liu X 2010 Effects of polarization on the de-excitation dark focal spot in STED microscopy J. Opt. 12 115707

[11] Galiani S, Harke B, Vicidomini G, Lignani G, Benfenati F, Diaspro A and Bianchini P 2012 Strategies to maximize the performance of a sted microscope Opt. Express 20 7362–74

[12] Kubby J A 2013 Adaptive Optics for Biological Imaging (Boca Raton, FL: CRC Press)

[13] Booth M J 2014 Adaptive optical microscopy: the ongoing quest for a perfect image Light Sci. Appl. 3 e165

[14] Deng S, Liu L, Cheng Y, Li R and Xu Z 2010 Effects of primary aberrations on the fluorescence depletion patterns of sted microscopy Opt. Express 18 1657–66

[15] Abramowitz M and Stegun I A 1964 Handbook of Mathematical Functions with Formulas, Graphs and Mathematical Tables (New York: Dover)