Axial superresolution with ultrahigh aperture lenses

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Abstract: We explore the current limits of the axial resolution of optical sectioning microscopy using a single lens, by combining the resolving power of novel 1.45 numerical aperture oil immersion lenses with superresolving binary aperture filters. We quantify the axial resolution brought about by the increase in semiaperture angle to $\alpha_{\text{max}} = 72.8^\circ$ and demonstrate an absolute gain in axial resolution through binary pupil filters. Implemented in a confocalized two-photon excitation microscope, the combination of both effectively improves the axial resolution to 330 nm full-width-half-maximum, which is by 34% over that of a standard 1.4 NA system.

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References and Links

1. Introduction

The generation of a small focal spot necessitates a large semiaperture angle \( \alpha_{\text{max}} \). Therefore, since the beginnings of light microscopy, lens manufacturers have been striving to provide the largest possible \( \alpha_{\text{max}} \) with minimal aberrations. For the optical sectioning microscopes, such as the scanning confocal and multiphoton excitation fluorescence, \( \alpha_{\text{max}} \) is even more important, since the axial extent of the focal spot scales largely with \((\sin \alpha_{\text{max}})^2\) \[1\].

Featuring an \( \alpha_{\text{max}} = 67.3^\circ \), the NA=1.4 oil immersion has the largest aperture of all diffraction-limited standard immersion lenses. For technical reasons, this angle is not easily surpassed in lens design, if a large Strehl-ratio is required across a large field of view. Parabolic mirrors readily deliver a nominal \( \alpha_{\text{max}} = 87^\circ \) but the aberrations associated with these elements preclude diffraction limited focusing \[2\]. Therefore one of the few viable approaches is to slightly increase the aperture angle while giving up field corrections. This has been realized in novel 1.45 NA oil immersion lenses (Carl-Zeiss, Göttingen, Germany) featuring a nominal semi-aperture angle increased by 5.5º, which is, to our knowledge, the highest aperture angle available to immersion microscopy at present. Our initial motivation for exploring these lenses was 4Pi-confocal microscopy, because, relying on the coherent superposition of two high aperture wavefronts, the benefits brought about by a slight increase in angle are augmented in this technique. We recently showed that in combination with dark-ring (DR-) binary aperture filters and multiphoton fluorescence excitation, these 1.45 lenses enable nearly spherical effective focal spots of \( \sim 150 \) nm full-width-half-maximum (FWHM) \[3, 4\].

In this Letter, we investigate the reduction of the axial extent of the point-spread-function (PSF) for single lens microscopy conditions by comparing the novel NA=1.45 lens with its standard 1.4 counterpart. Besides, we explore the effect of superresolving DR-filters in a confocal fluorescence microscope using two-photon excitation. While these filters have been of considerable interest in theory \[5-8\] and elegantly implemented \[9\], their superresolving effect has been verified only for a low NA=1.0, in which case an absolute improvement of resolution is not achievable \[9\]. Hence, actual axial superresolution remained yet to be demonstrated. In the end, we show experimentally that when combining the DR-filter with a NA=1.45 lens, the axial FWHM of the effective point-spread-function (PSF) of the two-photon excitation confocal microscope is reduced from a standard \( \sim 500 \) nm down to a remarkable \( \sim 330 \) nm.

2. Optical Setup

The setup (Fig. 1) utilizes a mode-locked Ti:Sapphire laser operating at \( \lambda_{\text{exc}} = 760 \) nm. In modern objective lenses, the aberration correction at this wavelength is similar to that in the visible range. The DR-filter imprints a zero-amplitude ring on the incident wavefront with inner and outer radii \( r_1 \) and \( r_2 \), respectively. The absolute values of these radii depend on the radius \( r_{\text{max}} \) defined by the NA of the lens \( L_1 \). An advantage of two-photon excitation is that the quadratic dependence of the fluorescence on the excitation intensity works against the higher order diffraction maxima associated with these DR-filters.

Z-responses are obtained by scanning a fluorescent polydiacetylene LB-monomayer with negligible axial thickness (< 5 nm) through the focal region of \( L_1 \). To exclude a chromatic longitudinal shift resulting from the disparity of the excitation wavelength to the central fluorescence wavelength of the layer, \( \lambda_F = 580 \) nm, a separate identical objective \( L_2 \) is employed as a confocalized imaging collector. \( L_2 \) is mounted on a piezo table such that the excitation and the detection PSF’s overlap. Confocality is realized by setting the pinhole radius to 0.65 of the fluorescence Airy disk diameter. A full aperture confocal microscope is
readily obtained by removing the DR filter. The counterpart standard aperture lens of \( \alpha_{\text{max}} = 67.3^\circ \) was a highly corrected NA=1.40 (PL APO, 100X oil, Leica, Wetzlar, Germany).

![Dark Ring (DR)](image)

**Fig. 1.** Schematic of the dark ring (DR) filter inserted into the excitation path of a confocal fluorescence microscope operating in transmission mode.

To probe the axial sectioning capability, an infinitely thin fluorescent plane is scanned through the focal volume to derive the z-response given by

\[
I(z) = C \int_{-\infty}^{\infty} [h_{\text{exc}}(z,r)]^2 [h_{\text{det}}(z,r) \otimes p(r)] r dr
\]

where \( h_{\text{exc}}, h_{\text{det}} \) denote the excitation and detection PSFs of \( L_1 \) and \( L_2 \), respectively; \( p(r) \) describes the detector pinhole [3]. All intensity distributions are calculated for linearly polarized excitation and randomly polarized detection [10]. The DR-confocal counterpart of \( I(z) \) is obtained by incorporating into \( h_{\text{exc}} \) a pupil function \( A(\theta) = 1 \) within \( \theta \leq \alpha_1 ; \alpha_2 \leq \theta \leq \alpha_{\text{max}} \) and \( A(\theta) = 0 \) otherwise. The parameter \( \theta \) is limited by \( \alpha_{\text{max}} = 67.3^\circ \) and \( 72.8^\circ \), for the NA=1.4 and the NA=1.45, respectively. \( \alpha_1 \) and \( \alpha_2 \) are bounded by the inner and outer radii, \( r_1 \) and \( r_2 \).

3. Results

The calculated z-response (Fig. 2a) indicates an increase in axial resolution as a consequence of the enlarged NA. The percentage gain in axial resolution of the NA=1.45 over the NA=1.40 objective lens is evaluated as follows: \( G = 1 - \left( \frac{F_{\text{Conf}}^{\text{NA}=1.45}}{F_{\text{Conf}}^{\text{NA}=1.40}} \right) \), with the parameter \( F_{\text{Conf}}^{\text{NA}} \) referring to the FWHM of the z-response of the corresponding objective lenses. The figure inset provides a truncated axial plot to highlight \( G=15\% \) resulting from \( F_{\text{Conf}}^{\text{NA}=1.45} = 370 \) nm.

The experimental z-response is shown in Fig. 2b. All the measured z-responses in this paper are systematically larger by 8-15\% compared to their theoretical counterparts, which is in accordance with all reported findings [11]. However, when comparing the NA=1.4 and the NA=1.45 lenses with each other, \( G=13\% \) is established, in good accordance with the calculation. This proves that the novel lens provides a real gain in aperture.
Next, we investigated the sharpening of the response through the DR-filter featuring a zero amplitude in the range of \( 0.25 \alpha_{\text{max}} < \alpha < 0.82 \alpha_{\text{max}} \). The filter divides the collimated excitation into a central low aperture beam and a cylindrical outer part. Interference sculpts the PSF in such a way that it diverts a part of the focal energy away from the main maximum, thus sharpening the maximum at the expense of spawning off higher order lobes. For points sufficiently remote from the focus, reduced multiphoton absorption and confocalization work in synergy to suppress these lobes. The design of the DR-filter has been chosen to balance between resolution gain and sidelobe height. Expanding \( r_2 \) and contracting \( r_1 \) favor the stronger participation of the marginal rays in the interference process. This squeezes the main maximum of the PSF even further, but at the same time unacceptably high lobes arise. To avoid this, a tolerance threshold of 10% of the maximal side lobe height has to be maintained as the \( r_1 \) and \( r_2 \) are chosen. The weak sidelobes in the PSF are witnessed both in the calculation (Fig. 3 a) and in the measurement (Fig. 3 b) as shoulders in the z-response, on top of which the sharper main peak resides.

![Theoretical and Experimental z-responses](image)

**Fig. 2.** (a) Theoretical z-responses of a two-photon confocal fluorescence microscope depicting the associated increase in axial resolution as the NA is expanded from 1.40 (bold) to 1.45 (thin). The experimental counterparts in (b) essentially confirm the predicted improvement in axial sectioning. The increase is magnified in the insets plotted with truncated axial coordinates.
In the DR-mode, switching to a NA=1.45 lens leads to $G=10\%$ in the calculation and $12\%$ in the measurement, again in good agreement with each other. Hence the $5.5^\circ$ increase in numerical aperture leads to the same gain of about $10\text{-}15\%$, both in theory and experiment. Interestingly, this is irrespective of whether the DR-filter is applied or not. However, when combining the DR-filter and the NA=1.45 lens we find experimentally that the main maximum is significantly sharpened up compared to that of a regular two-photon confocal microscope using a NA=1.4 lens, resulting in a $F^\text{DR}_{\text{NA}=1.45} = 330$ nm. The inset in Fig. 3b emphasizes this narrowing of the main maximum by $34\%$.

![Figure 3](image_url)

**Fig. 3.** Z-responses of a two-photon DR-confocal microscope. (a) Theoretical responses for NA=1.40 (thin) and NA=1.45 (bold). Panel (b) shows the experimental counterparts to (a). The combination of DR-filter and NA=1.45 lens sharpens the z-response by $34\%$ with respect to that of a standard NA=1.40 confocal microscope, resulting in a full-width-half-maximum of 330 nm.

We note, however, that in imaging applications the full exploitation of the increased aperture will also be challenged by an increased sensitivity of these lenses to sample induced aberrations, although one could counteract these aberrations with active optical elements, that also create the DR-filter at the same time [9]. There are also other reasons why the aperture increase reported herein is important. Apart from the fact that it increases the collection efficiency by $25\%$, even a slight increase in aperture should facilitate the generation of...
orthogonal field components in the focal region of the lens [10, 12-14]. Illumination with, for example, linearly x-polarized light strongly augments the y- and in particular the longitudinally (z-) oriented focal field components, which have implications for single molecule spectroscopy and related studies. The orthogonal field components could further be enhanced through the implementation of annular apertures and by a 4Pi-confocal arrangement, in which case nearly pure longitudinally (z-) polarized focal field components or imaging modes could be produced.

4. Conclusion

In summary, besides giving experimental evidence for an effective increase in axial resolution with novel 1.45 numerical aperture oil immersion lenses, we provided, to our knowledge, the first experimental proof of axial superresolution through binary amplitude pupil filters. The combined action of increased aperture and suitable pupil filters reduces the FWHM of the axial response from typically 500 nm to 330 nm which is a new lower benchmark for single lens imaging relying solely on fluorescence excitation.

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