Wavefronts in the focus of a light microscope

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Summary
We report the visualization of the wavefronts of light in the focal region of an objective lens. The focused wavefronts are measured for the highest available numerical aperture, i.e. 1.4 oil immersion. The measurement is accomplished by scanning a scattering probe through the focal region of a lens illuminated by plane waves. The scattered light is coherently added to an external reference beam and focused into a point-like detector. The registered signal allows the determination of the three-dimensional shape of the wavefronts in the focal region and their evolution in time.

1. Introduction
In spite of the unrivalled success of electron and probe microscopes in resolution, focusing light microscopy remains highly attractive for many applications in biology and material sciences (Wilson, 1990; Herman & Lemasters, 1993; Pawley, 1995). Recent achievements in laser physics and optics enabled the development of laser scanning microscopy (Sheppard & Wilson, 1978; Brakenhoff, 1979), which now also includes two-photon (Denk et al., 1990) and three-photon fluorescence imaging modes (Hell et al., 1996). Modern techniques further include lifetime imaging (Marriott et al., 1991; Lakowicz et al., 1992; Müller et al., 1995; So et al., 1995), Raman confocal microscopy (Puppels et al., 1990), photobleaching-recovery techniques (Wedekind et al., 1994) and fluorescence resonance energy transfer microscopy (Clegg, 1995). The large variety of imaging modes testifies to the versatility of the focusing approach.

From the earliest stages on, the perfection of focusing has been a major point of concern in far-field light microscopy (Abbe, 1873). Perfect focusing is accomplished by a segment of a wavefront that is entirely spherical (Fig. 1). Deviation from the spherical shape, i.e. aberrations of the wavefront, generally result in a more blurred focal intensity distribution (Born & Wolf, 1991). The ability of the objective lens to produce spherical wavefronts in the sample ultimately determines the resolution of a far-field microscope. Investigating the shape of the wavefront is of utmost importance for far-field imaging. Hence, the behaviour of waves in the focal region of the lens has received great attention in the theoretical literature; a detailed account of this behaviour is given in the volume by Stannnes (1986). Furthermore, a good understanding of the focusing process is fundamental for overcoming the present resolution limits in the far-field (Hell, 1992, 1994; Hell & Wichmann, 1994; Schrader & Hell, 1996).

A possible approach to quantify the focal process is to measure the focal intensity distribution (Brakenhoff et al., 1979; Hell et al., 1994). The intensity distribution in the focus is mathematically described by the intensity point-spread-function (PSF). However, the PSF is the end result of focusing and does not give a direct quantification of the aberration. There are several good methods described in the literature for measuring the wavefronts produced by a lens, most of which are interferometric. The standard method is based on a Twyman–Green interferometer (Twyman & Green, 1916). In such a set-up, the wavefronts from a spatially coherent light source are split into a reference beam and a signal beam illuminating the objective lens. Whereas the reference beam is directed towards a plane mirror, the signal beam is focused by the lens. To quantify the performance of the lens, a spherical mirror is placed in the focal region, the role of which is to reflect the wavefronts back into the lens and onto the beam splitter (Twyman, 1921; Kingslake, 1927). The beam splitter recombines the back-reflected wavefronts from the lens and the reference mirror, and directs them towards a screen where the interference pattern can be observed. This method allows the precise determination of the wavefront and meets most of the requirements in lens testing. However, one cannot visualize the wavefronts in the focal region with this method because the screen is in a plane that is optically conjugate to the exit pupil. As a result, the change in the amplitude and phase of the wavefronts in the focal region are not quantified or visualized. The latter, however, is the aim of the method described herein.

Our method is also based on a Twyman–Green interferometer, but it enables what is to our knowledge the first direct visualization and measurement of the focused waves.
Fig. 1. Scheme of the wavefronts produced by a lens. Distances between the wavefronts correspond to one wavelength, and when referring to Fig. 3 to half a wavelength. In this scheme they are enlarged with respect to the dimensions of the lens.

In contrast to the prior art, we probe the focused waves with a scattering point-like object rather than a curved mirror and measure the interference signal in a point-like detector. The point-like detector is optically conjugated to the focal point of the lens (Fig. 2). For every coordinate in the focal region the light that is scattered from the point-like object carries both the information of the amplitude and the phase at this particular coordinate. When adding the scattered light with the focused reference beam in the detection pinhole, we can determine both the amplitude and the relative phase of the light in the focal region.

2. Theory

The theoretical description of our set-up is straightforward. One can imagine Fig. 2 as a confocal microscope with an added-on interferometric reference arm. Hence, the signal in the detector \( I_d \) is given by the coherent superposition of the confocal signal (Wilson, 1990) of a point-like object \( c_I \hat{h}^2(x,y,z) \) and a reference wave \( a_e e^{i\phi} \) of arbitrary, yet well-defined amplitude and phase:

\[
I_d(x, y, z) \propto |c_I \hat{h}^2(x,y,z) + a_e e^{i\phi}|^2
\]

\( \hat{h}(x,y,z) \) is the amplitude point-spread-function and denotes the electric field. The variables \( (x,y,z) \) denote the spatial coordinates in the focal region having an origin in the geometric focus. The constant \( c_I \) is a normalization parameter considering, for example, the finite transmission of the lenses; \( \hat{h}(x,y,z) \) contains both the information of the amplitude and the relative phase of the electric field in the focal region. A point-like scattering object probes this function.

The role of the reference beam is to extract the phase information that is contained in \( \hat{h}^2(x,y,z) \). The detector signal depends on the location \( (x,y,z) \) of the scattering point-like object in the focus and reveals the information about the relative phase and the amplitude in the focal region of the lens. The points resulting in constructive interference with the reference beam appear bright, whereas those with destructive interference appear dark.

There are several methods to calculate the focal electric field \( \hat{h}(x,y,z) \). We follow Richards & Wolf (1959) and write the electric field as \( \hat{h}(x,y,z) = (e_x, e_y, e_z) \) with \( e_x = -iI_0 + I_3 \cos 2\phi \), \( e_y = -I_3 \sin 2\phi \) and \( e_z = -2I_1 \cos \phi \). The angle \( \phi \) defines the azimuth angle between the polarization of the incident illuminating field and the direction of observation. The parameters \( I_0, I_1, I_2 \) are integrals over the lens aperture with \( I_1, I_2 \) vanishing for lower apertures. For simplicity, we consider only the case of \( \phi = \pi/2 \). We also prefer to calculate the detector signal \( I_d \) for the highest numerical aperture (nominal aperture of NA = 1.4, oil immersion, refractive index \( n = 1.518 \)) and for a wavelength of \( \lambda = 632.8 \) nm, as these were also our experimental parameters. In our calculations we applied a numerical aperture of 1.35 rather than of 1.4 since measurements indicated

Fig. 2. Set-up for measuring the wavefronts in the focal region of a lens.
this numerical aperture to be more appropriate for the Leica, Plan Apo, 100× lens in use (Wilson & Juškaitis, 1995). In a practical situation, the constants $c_f$ and $d_f$ also depend on the transmission of the lens, and the strength of the reference beam, respectively. In an experiment, one can arbitrarily choose the strength of the reference beam $d_f$ with respect to the focal signal $c_f$. The same applies to the relative phase $\psi_f$.

Figure 3a shows the calculated detector signal $I_d$ for a given phase difference, which is that the light emanating from the focal point interferes destructively with the reference beam. A $c_f/d_f$ is chosen so that the signal matches the measured data of Fig. 3b. (The measurement of the wavefronts will be described in the experimental section of this paper.) In Fig. 3a, the focal wavefronts are well pronounced. One can think of a wavefront as the focal coordinates with the same relative phase. In the outer regions of the focus, the distance between the wavefronts is $\lambda/2n$, i.e. 208 nm. In the vicinity of the geometric focus, the focused wavefronts deviate slightly from the spherical shape and the distance between the wavefronts is larger than $\lambda/2n$.

3. Experiment

The experimental set-up consists of a confocal stage scanning interference microscope based on a Twyman–Green interferometer as shown in Fig. 2. Scanning is accomplished by a piezoelectric 3D-positioner, capable of moving specimens with a precision of 10 nm in three
wavefronts are clearly visible. One can imagine the wavefronts coming from the left and propagating to the right. In that sense, Fig. 3b can be seen as a ‘snapshot’ of the focusing process. The similarity between Figs. 3a and b is apparent.

Equation (1) suggests that the amplitude of the signal \( I_d \) contains both the intensity of the reference beam as well as the fourth power of the electric field of the actual wavefront. The retrieval of the focal amplitude is not as straightforward as that of the relative phase. One could approximate the real amplitude of the wavefronts by taking the fourth root of the detector signal, of course. Here, we do not follow this approach, rather we concentrate on the charge of the phase of the wavefronts. All the points in the focal region that are in constructive interference with the reference beam are bright, whereas those with destructive interference appear dark. By changing the phase of the reference beam \( \psi_0 \), other coordinates of equal phase appear bright, i.e. the other wavefronts become visible. We changed the phase \( \psi_0 \) by applying a different voltage to our piezoelectrically driven reference mirror. Figure 4 shows three different XZ-wavefront images with three different phases \( \psi_0 \). They can be imagined as a succession of temporally evolving wavefronts. In fact, we recorded an even denser succession of wavefront images. By displaying the wavefronts quickly one after the other we were able to visualize the propagation of the wavefronts in the focal region (Schrader & Hell, paper presented at Conference on 3D Imaging Sciences in Microscopy, Oxford, April 1996, unpublished).

We also recorded XY-images of the wavefronts, i.e. cuts perpendicular to the optical axis. When taking an XY-image in a plane such as the one marked with P (see Fig. 1), one obtains ring-shaped areas of equal phase. XY-images are displayed in Fig. 5, each of them recorded at different axial positions Z. The planes were 40 nm apart in Z. One can also think of the plane containing P as being moved towards the focus in steps of 40 nm, in Fig. 5 from top to bottom. The change in the phase gives a feel for the convergence of the spherical wavefronts into the focal point. A quick successive display also allows the visualization of the converging wavefronts. Figures 5a and f display similar phase distributions because five steps of 40 nm in Z give an axial shift of about half of the wavelength.

4. Discussion and conclusion

Our experiments show that it is possible to visualize and measure the wavefronts in the focal region of a high-numerical-aperture lens. The wavefronts are probed by means of a subresolution scattering gold bead that is precisely scanned through the focal region of the lens in an interferometric confocal set-up. Changing the phase of the reference beam enables the visualization of the temporal evolution of the wavefronts.
When comparing the theory with the experiment (Fig. 3a and b), we find a good agreement between the data. This is displayed in Fig. 3c where the calculated and experimental detector signals are compared. Our experiment reveals the shape of the wavefronts and the distance between them. Figure 3b gives a feel for the aperture of the used lens and, in principle, allows a determination of the effective aperture. In general, the spherical shape of the wavefronts is nicely preserved in the focus, as it is expected from a well-corrected lens.

In the experimental data (Fig. 3b), one can notice parallel intensity lines in the out-of-focus area close to the focal plane. These lines are absent in the theoretical wavefront image. In these regions, the scattered light from the gold bead is very weak because it is out of focus. We think that the parallel lines stem from a residual reflection from the cover glass/oil interface that interferes with the reference beam. In the focus, these reflections are outweighed by the strong signal from the gold bead. When viewing Fig. 3, one also has to bear in mind that according to Eq. (1), the ‘wavefronts’ are basically proportional to the square of the intensity of the real focal wavefronts, so that the signal at the outer region is considerably suppressed.

Our method of measuring the wavefronts offers new opportunities for quantifying the focusing performance of a lens. For instance, one could induce aberrations in a controlled manner in order to study the relation between the focal wavefronts and the intensity PSF which can be measured simultaneously (Hell et al., 1993). Furthermore, one could also measure wavefronts of different wavelengths of light, i.e. with different laser lines. Even more interesting, though technically challenging, could be measurements of the wavefronts of pulsed laser light, for instance pulses of the order of 10 fs or shorter. In this case, the coherence length of the light is of the order of the focal extent. With pulses of 5 fs one can associate a coherence length of \( \approx 1.5 \mu m \), which is about the focal extent along the optical axis. The wavefront behaviour of these pulses is expected to deviate slightly from the one shown here (Bor & Horvath, 1992) and yield a resolution that is improved by 10–20% (Gu & Sheppard, 1994).

Finally, we note that the obstruction of the reference path in Fig. 2 and simultaneous removal of the pinhole allows the measurement of the intensity of the light in the focus. In combination with our method of measuring the wavefront, one can completely map the focus in amplitude and phase.

Fig. 5. XY-wavefront images separated by 40 nm in the z-direction. Starting from one interference maximum at about 600 nm distance to the focus the sequence shows the wavefront alter every 40 nm step until the next maximum is reached.
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References


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