Measurement of specimen-induced aberrations of biological samples using phase stepping interferometry

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Summary
Confocal or multiphoton microscopes, which deliver optical sections and three-dimensional (3D) images of thick specimens, are widely used in biology. These techniques, however, are sensitive to aberrations that may originate from the refractive index structure of the specimen itself. The aberrations cause reduced signal intensity and the 3D resolution of the instrument is compromised. It has been suggested to correct for aberrations in confocal microscopes using adaptive optics. In order to define the design specifications for such adaptive optics systems, one has to know the amount of aberrations present for typical applications such as with biological samples. We have built a phase stepping interferometer microscope that directly measures the aberration of the wavefront. The modal content of the wavefront is extracted by employing Zernike mode decomposition. Results for typical biological specimens are presented. It was found for all samples investigated that higher order Zernike modes give only a small contribution to the overall aberration. Therefore, these higher order modes can be neglected in future adaptive optics sensing and correction schemes implemented into confocal or multiphoton microscopes, leading to more efficient designs.

Introduction
A confocal microscope in fluorescence mode probes the three-dimensional (3D) distribution of fluorescent molecules within the sample. In the ideal case, the excitation light is focused to a diffraction-limited focal spot where fluorescent molecules can be excited. The emitted fluorescence light is focused on a confocal detector pinhole, which rejects out-of-focus light, and leads to the well-known optical sectioning ability of the confocal microscope. This is especially useful for 3D-imaging of thick biological specimens (Wilson & Sheppard, 1984; Wilson, 1990; Pawley, 1995).

Apart from the distribution of the fluorophore that is imaged, a typical biological specimen shows a variation in refractive index. As the light propagates within the sample the variation in refractive index deforms the wavefront resulting in a deviation from its ideal shape. This is known as aberration. Specimen-induced aberrations can be caused by variations of the refractive index, a refractive index mismatch between the sample and the embedding medium (Hell et al., 1993; Booth et al., 1998) or the use of an inappropriate immersion fluid.

In the presence of aberrations the intensity distribution of the spot probing the sample does deviate from the ideal, diffraction-limited case and resolution and signal intensity are reduced (Wilson & Carlini, 1989; Hell et al., 1993). Signal intensity is an important issue in fluorescence microscopy because bleaching occurs with many dyes and the energy deposited within the sample should be kept at a minimum (Pawley, 1995).

The principle of adaptive optics to correct for aberrations was first applied in astronomy (Hardy, 1998). Later it was suggested to use adaptive optics in confocal and multiphoton microscopy (Booth et al., 1998, 2002; Neil et al., 2002b; Sherman et al., 2002). The idea is to feed the optical system with a pre-aberrated wavefront that cancels out the aberrations present in the system and restores diffraction-limited imaging. Such adaptive optics systems comprise a wavefront-sensing device (e.g. a Shack–Hartmann sensor (Platt & Shack, 2001), an interferometer-type sensor or a modal sensor (Neil et al., 2000a)) and an active correction element (e.g. a deformable mirror or a spatial light modulator (SLM)). If one wants to design such a system or estimate its benefits for standard applications, it is necessary to know the types and amount of aberration that one has to correct for in a standard biological sample.

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†Note that the normalization and indexing are different between the definitions in Born & Wolf (1983) and Noll (1976). We refer to the definition given in Noll (1976).
specimen. This was the motivation behind building the interferometer-type system presented here.

**Experimental system**

A schematic drawing of the set-up is shown in Fig. 1. The expanded He–Ne (632 nm) laser beam illuminating the set-up was split into a reference path and an object path. A rotation of the λ/2-plate in front of the polarizing beamsplitter (PBS) permits adjustment of the relative intensities of the two paths. The Zeiss LD-Achroplan, 40×, 0.6 NA, which was used as a condenser, has a correction ring to adjust for the thickness of the microscope slide, thereby minimizing spherical aberration. We used both a Zeiss 20 Plan-Neofluar, 0.5 NA (dry lens without coverglass correction) and an Olympus 20× UPlan-Apo, 0.8 NA, oil-immersion as objective lenses. When the object beam has traversed the specimen it is made to interfere with the reference beam and the resulting interference pattern is recorded on a CCD camera. The image plane of the CCD is conjugate to the pupil plane of the objective lens. The phase stepping unit placed in the reference beam path takes the form of a flat mirror shifted by a calibrated piezo drive. This allows us to change the relative phase between reference and object beams in well-defined steps. This phase stepping is synchronized with the CCD camera and permits the recording of digital interferogram images at the video rate of 25 Hz using a framegrabber fitted to a Linux PC. The specimen is attached to a piezo-driven stage that can be positioned in three dimensions, and feedback control prevents long-term drift of the sample (P-611 nanocube, Physik Instrumente, Germany).

Furthermore, the object path can be switched to operate as a conventional transmission microscope using the additional camera and illumination elements shown in Fig. 2. This bright-field illumination mode is used to find regions of interest within the sample prior to the data recording in the interferometer mode and the alignment is such that the focal plane of the transmitted light image coincides with the focal spot of the interferometer.

Because we are concerned with aberrations in confocal microscopes we have to verify whether this experiment actually models a confocal microscope set-up. The phase stepping interferometer works in transmission mode whereas a typical confocal microscope employs epi-detection in which the emitted fluorescence light is collected by the same lens. The two set-ups are shown for comparison in Fig. 3. If the focus is set to the bottom of the specimen, the aberrations introduced within the two beam paths are equivalent because the wavefront leaves the sample below the focus, experiencing no further aberration. Similarly, a focal position at the top of the specimen below the coverslip is equivalent to an epi configuration focused to the top of the specimen from below. Only these two configurations deliver results identical to the epi set-up of a...
confocal or multiphoton microscope. This is not a severe limitation. We are interested in the maximum aberration that we would have to correct for in an adaptive optics system.

Equivalent aberration problems are present in two-photon (Denk et al., 1990) or multiphoton microscopes. However, for an actual implementation within a confocal microscope it is feasible to correct both paths of the set-up using a single correction element (Booth et al., 2002).

### Data acquisition

The wavefront recorded for one particular position of the sample contains three contributions to the total aberration, namely the static aberrations of the optical system, the static specimen-induced aberrations and the field-dependent specimen-induced aberrations. Static aberrations of the optical system may originate from misalignment or slight imperfection of the optics. Specimen-induced static aberrations can be caused by focusing into media with mismatched refractive indices, and the field-dependent specimen-induced aberrations result from the variation in refractive index of the sample.

We are interested in measuring both types of specimen-induced aberrations. It is feasible to separate all these contributions to the aberration by a two-step calibration. First, a reference wavefront 1 is recorded from a microscopic slide with a coverslip on top without any sample, ensuring no additional static specimen-induced aberrations are present. For this measurement the focus is set directly below the coverslip and the correction ring of the condenser lens is adjusted to minimize the remaining aberrations. In a second step the sample with the actual biological specimen within a water-based solution is inserted without alteration of the optical setting of the lenses. Then a reference wavefront 2 is recorded at a position where the light traverses the homogeneous part of the sample next to the specimen.

During the next and final step of the experiment the stage is scanned in the x–y plane and wavefronts are recorded in a raster fashion. Now the difference between the reference wavefronts 1 and 2 gives the static specimen-induced aberration whereas the difference between the wavefronts recorded in the scan across the sample and reference wavefront 2 gives the field-dependent component of the specimen-induced aberrations.

With regard to the static component of the specimen-induced aberration, it should be mentioned that this is directly dependent on the difference between the two absolute measurements of the specimen slide and a reference slide. This assumes that both the coverslip and the microscopic slide of the measured specimen have identical thickness and refractive index. Despite nominal standardization, these glass slides may differ slightly.

For the data shown in this paper we recorded 256 wavefronts corresponding to the 256 positions within a 16 × 16 grid of focal positions for each specimen. To illustrate the scanning process, a transmitted light image of a mouse blastocyst sample is shown in Fig. 4. One scan takes less than 5 min. The acquisition of the wavefront at each point involves the recording of three interferogram images at relative phase steps of 0°, 120° and 240°, which are then processed to reconstruct the wavefront as detailed in the next section.

### Data processing

The complex wavefront, \( P(x,y) \), in the pupil plane of the objective can be expressed in terms of its amplitude \( A(x,y) \) and phase \( \psi(x,y) \) as

\[
P(x,y) = A(x,y) \exp(j \psi(x,y)).
\]  

One wavefront measurement consists of a set of three interferograms. Assuming unity amplitude for the reference beam each interferogram has the form

\[
I(x,y,\Delta \psi) = 1 + A^2(x,y) [1 + 2 \cos(\psi(x,y)) + (\Delta \psi)],
\]  

where \( \Delta \psi \) is the phase-shift introduced in the reference arm. Because three interferograms \( I_1 = I(x,y,0) \), \( I_2 = I(x,y,2\pi/3) \) and \( I_3 = I(x,y,4\pi/3) \) for known values of \( \Delta \psi \) are recorded one can solve for the phase of the wavefront:

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Fig. 3. Comparison between the confocal LSM and the interferometer set-up. The two beam paths are equivalent when focused to the bottom of the specimen.
The phase is calculated for every pixel position \((x, y)\) and yields the wrapped phase \(\phi(x, y)\), which is the absolute phase of the wavefront \(\psi(x, y)\) modulo \(2\pi\). A Fourier fast transform (FFT)-based phase unwrapping technique (Ghiglia & Pitt, 1998) is now applied to recover the unwrapped phase \(\psi(x, y)\) or its equivalent \(\psi(r, \theta)\) in polar coordinates. In order to describe the aberration present in the wavefront, it is convenient to use a Zernike-mode representation. The Zernike polynomials \(Z_i(r, \theta)\) (Noll, 1976; Born & Wolf, 1983) are a set of functions that are orthogonal over the unit circle. For their definition, see the Appendix. One can represent the phase function \(\psi(r, \theta)\) as a series expansion in Zernike-polynomials:

\[
\psi(r, \theta) = \sum_{i=1}^{\infty} M_i Z_i(r, \theta)
\]

(4)

where the modal coefficients \(M_i\) of the wavefront are given by:

\[
M_i = \frac{1}{\pi} \int_0^{2\pi} \psi(r, \theta) Z_i(r, \theta) r d\theta dr.
\]

(5)

The set of Zernike-coefficients \(M_i\) describes the aberration function \(\psi(r, \theta)\). Some Zernike-modes correspond to the classical terms of aberrations, for instance astigmatism (modes 5 and 6) or coma (modes 7 and 8). In our data analysis we characterized the wavefront by the Zernike modes 2–22.

The modes 2, 3 and 4 have the common property that they create geometrical distortions of the image but do not compromise resolution or signal intensity. Mode 2 (tip) represents a linear variation of the wavefront in the \(x\)-direction whereas mode 3 (tilt) is a linear wavefront slope in the \(y\)-direction. These correspond to lateral displacements of the focal spot in the \(x\)- and \(y\)-directions, respectively. Similarly, mode 4 (defocus) alters the axial position of the focal spot and has no influence to the shape of the point spread function (PSF). In an epifluorescence confocal microscope these modes are self-correcting because of the dual pass nature of this configuration. Therefore, within an adaptive optics system that aims to restore diffraction-limited imaging it is not necessary to correct for these modes.

**Results**

We chose water-based preparations of mouse oocyte cells and blastocysts and *C. elegans* as biological test specimens. Examples of measured wavefronts are shown in Fig. 5. The colour of the images represents the phase and the brightness corresponds to the amplitude. On Fig. 5(a), a wavefront corresponding to

![Fig. 4. Transmitted light image of the mouse blastocyst sample. The recording of the wavefront at different raster positions is illustrated by the superimposed green dots. A total of 256 wavefronts were recorded at a 16 × 16 grid. The size of the scan area indicated by the red frame is 130 \(\mu\)m × 130 \(\mu\)m.](image)

**Fig. 5.** Examples for measured complex pupil plane wavefronts. The colour represents the phase and the brightness corresponds to the recorded intensity. (a) Wavefront recorded beside the cell (objective NA = 0.5, dry; condensor NA = 0.6). (b) Aberrating region of the sample (mouse blastocyst, objective NA = 0.5, dry; condensor NA = 0.6). (c) Aberrating region of the sample (mouse oocyte, objective NA = 0.8, oil; condensor NA = 0.6). The circles indicate the limiting aperture of the system.
a focal position away from the cell is shown. Here the lens positions and the coverglass correction have been adjusted to minimize phase variation. This adjustment is accounted for in the calibration steps. Figure 5(b,c) show examples of aberrated wavefronts. For Fig. 5(a,b) an objective lens with an NA of 0.5 was used. As this NA is lower than that of the condenser lens (0.6) the pupil plane image has sharp borders, indicated by the circles. These circles mark the nominal aperture of the system over which we performed the Zernike mode fitting. Figure 5(c) was recorded using a lens with an NA of 0.8, which was higher than that of the condenser. Small amounts of light are refracted into angles greater than the aperture angle of the condenser but are still accepted by the larger aperture of the objective. This is indicated by intensities outside the circle.

If the Zernike modal content extracted from the wavefront is plotted against its position within the 2D scan of the specimen we obtain a Zernike pseudo-image for that particular mode. These plots are shown for the mouse oocyte sample in Fig. 6.
Here the sum of the static and field-dependent fractions of the specimen-induced aberration are plotted, and the static aberration introduced by the optical system was removed using the described two-step calibration method. The first image on the first line of Fig. 6 shows the sum of the absolute values of the coefficients 4–22 and corresponds to the total aberration apart from tip and tilt. The Zernike coefficient $M_2$ (tip) corresponds to a linear variation of the wavefront in the horizontal direction whereas $M_3$ (tilt) represents a linear slope in the vertical direction. All values are given in units that are defined such that one unit is equivalent to a wavefront standard deviation of one radian (see Appendix). The Zernike charts of the mouse oocyte showed rather simple symmetry because of the spherical-like shape of the cell. Plots of tip and tilt are similar because of the symmetry of the sample and the fact that the corresponding Zernike polynomials are identical apart from a rotation of 90° about the origin.

Note also the similarity between the two astigmatism modes 5 and 6. The Zernike polynomials for these modes are identical except for a rotation of 45°. The mean and standard deviation across the field of view for the extracted Zernike coefficients for 256 measured wavefronts.

**Fig. 8.** Zernike-mode plots for the coefficients 2–22 of the mouse blastocyst sample. For these images 256 wavefronts were recorded and the Zernike modal content was extracted as described in the text. Objective lens: Zeiss Plan-Neofluar 20×, 0.5 NA, dry. Condenser lens: Zeiss LD-Achroplan, 40×, 0.6 NA, correction ring. The scanned area was 130 µm × 130 µm.

**Fig. 9.** Mouse blastocyst sample. Mean and standard deviation across the field of view for the extracted Zernike coefficients for 256 measured wavefronts.
of each mode are shown in Fig. 7. The large value found for the mean of the spherical aberration (mode 11) agrees with theoretical expectations from focusing into a sample of mismatched refractive index (Hell et al., 1993; Booth et al., 1998; Booth & Wilson, 2000). A decrease in the standard deviation of measured Zernike modes occurs with rising order. In addition to this decrease, superimposed step-like changes of the standard deviation can be seen. This interesting effect can be understood by considering the definition of the Zernike polynomials (see Appendix). The single indexing scheme maps to the two independent azimuthal and radial indices of the Zernike modes. The wavefronts measured close to the centre of the sphere-like sample are expected to contain mainly radial spatial frequencies. Steps are visible between coefficients (6, 7), (10, 11), (15, 16) and (21, 22). These are exactly those pairs of indices i for which the radial frequency of the

Fig. 10. Zernike-mode plots of the C. elegans sample, coefficients 2–22. For these images the Zernike modal content was extracted from 256 wavefronts. Objective lens: Olympus 20× UPlanApo, 0.8 NA, oil immersion. Condenser lens: Zeiss LD-Achroplan, dry, 40×, 0.6 NA, correction ring. The scanned area was 100 µm × 100 µm.

Fig. 11. Nematode C. elegans sample. Mean and standard deviation of the Zernike coefficients across the field of view for 256 measured wavefronts. For this sample the calibration included one reference wavefront. Therefore, the field-dependent specimen-induced aberrations are shown. Static system- or specimen-induced aberrations are not included.
polynomials changes. The rather constant part of the step-like decrease in the standard deviations of the Zernike modal content correspond to changes in the azimuthal spatial frequencies, which are under-represented in an object of this symmetry.

The next sample, a mouse blastocyst specimen, was less symmetric and showed more complex patterns within the extracted Zernike modes. Several cavities filled with liquids of a different refractive index are typical for this type of specimen. Figure 4 displays a bright-field image of this sample where the focus is adjusted to the middle of the specimen. The scanning process is indicated as well. Plots for the Zernike modes 2–22 of the blastocyst sample are depicted in Fig. 8. The sum of the static and field-dependent components of the aberration is displayed here. Note that again the variation of the coefficients declines with increasing order of the successive Zernike modes (Fig. 9).

As with the previous sample, coefficient number 11 (spherical) again shows a significant offset as expected. For both mouse specimens the Zeiss Plan-Neofluar 20×, 0.5 NA objective lens was used. This dry lens does not have a coverslip correction but the correction ring of the condenser lens was adjusted during the calibration. Therefore, the additional spherical aberration introduced by the coverslip of the sample was compensated. Only the spherical aberration originating from the liquid layer of the specimen was added to the total aberration measured.

For the last sample, a C. elegans specimen, the experimental conditions were slightly different: an Olympus 20× UPlanApo, 0.8 NA, oil-immersion, was used as the objective lens and a one-step calibration in respect to the reference wavefront 2 within the specimen slide was performed. This means that the aberration data shown in Figs 10 and 11 show the field-dependent fraction of the specimen-induced aberration only: the static part caused by focusing into the sample is not included. Therefore, the mean spherical aberration (mode 11) was much lower compared with the other samples. Again, we see that the magnitude of the variations declines as the index of the modes increases.

Discussion and conclusion

We have measured the specimen-induced aberrations for biological samples using a phase stepping interferometer. The Zernike mode representation is a useful technique to describe the aberrations.

The same general behaviour of declining higher Zernike coefficients was observed for all samples investigated. This suggests that it is convenient to implement an adaptive optics system into a confocal microscope where the sensing and correction scheme is based on the direct handling of Zernike modes. Higher order modes of the wavefront can then be neglected leading to more efficient designs. Within the static components of the specimen-induced aberration the spherical aberration (mode 11) dominated and was in the range of up to 0.5 Zernike units. Within the field of view, standard deviations up to about 0.4 Zernike units for low-order modes where measured.

This suggests that it is desirable to have a field-dependent correction of the aberrations for these low order modes rather than just an adaptive compensation for the spherical aberration caused by focusing deep into the sample.

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References


Appendix: Zernike polynomials

Several definitions of the Zernike polynomials with different normalization factors and mapping schemes for the indices exist. Therefore, the version we used for the extraction of the Zernike coefficients from the wavefront is included here. We preferred a single index $i$ but other authors (e.g. Noll, 1976; Born & Wolf, 1983) use two indices that relate to the azimuthal and radial orders. The polynomials may then be defined as (Neil et al., 2000a):

$$Z_n^m(r, \theta) = \begin{cases} 
\sqrt{2}R_n^m(r) \sin(-m\theta) & \text{if } n < 0, \\
0 & \text{if } n = 0, \\
\sqrt{2}R_n^m(r) \cos(m\theta) & \text{if } n > 0,
\end{cases}$$

$$R_n^m(r) = \sqrt{n+1} \sum_{s=0}^{(n-m)/2} \frac{(-1)^s (n-s)!}{s!(n+m)/2-s!((n-m)/2-s)!},$$

where the indices $m$ and $n$ are restricted to the conditions $n \geq |m|$ and $n - |m|$ is even. This is, apart from the indexing, equivalent to the definition of Noll (1976) and has the advantage of a normalization such that the mean is zero over the unit circle and the variance is unity (except for $n=0$ where it is zero). All data in this paper are given in Zernike coefficient units according to the definition in Eq. (6). By calculating the wavefront using Eqs (4) and (6) we directly obtain the aberration of the wavefront over the normalized pupil of the system in radians. The orthogonality relation does not depend on $n$, in contrast to the definition in Born & Wolf (1983). The rules to map the double indices to the single index $i$ are: $i$ starts at 1 for $n = 0$ and rises firstly with $n$, then all allowed values of $m$ are ordered with rising magnitude where the positive values come first. The first 22 polynomials $Z_n^m(r, \theta)$ are listed in the Table A1 below:

<table>
<thead>
<tr>
<th>$i$</th>
<th>$n$</th>
<th>$m$</th>
<th>Aberration term</th>
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<tr>
<td>1</td>
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<td>0</td>
<td>piston</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>$2r \cos(\theta)$</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
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<td>$2r \sin(\theta)$</td>
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</tr>
<tr>
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</tr>
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<td>$2\sqrt{3}(3r^3 - 2r) \cos(\theta)$</td>
</tr>
<tr>
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