Optical Imaging in Biology and Medicine

Master in Photonics & Europhotonics Master Program

Adaptive Optics for Microscopy

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Abstract

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TODO!

- **introduce abbreviations for Adaptive Optics (AO) and Adaptive Optics Microscopy (AOM) in the intro and then use them through the text**

Contents

1 Introduction

actual techniques in the second part, methods
[4]

The performance of these microscopes is often compromised by aberrations that lead to a reduction image resolution and contrast.

These aberrations may arise from imperfections in the optical system or may be introduced by the physical properties of the specimen.

The problems caused by aberrations can be overcome using adaptive optics, whereby aberrations are corrected using a dynamic element, such as a deformable mirror.

This technology was originally conceived for the compensation of the aberrating effects of the atmosphere and was first developed for military and astronomical telescopes.

Adaptive optics systems have also been introduced for other applications such as laser beam shaping, optical communications, data storage, ophthalmology and microscopy.

[3]

Optical microscopes have long been essential tools in many scientific disciplines, particularly the biological and medical sciences. Conventional widefield microscopes—encompassing transmission, phase contrast and fluorescence imaging modes—are the workhorses of many laboratories. Over the last 25 years, researchers have also made significant developments in 3-D imaging using scanning laser microscopes. This progress started with the confocal microscope, which provides 3-D resolution by using a pinhole to exclude out-of-focus light. Rather than produce a whole image simultaneously, these microscopes scan a laser spot through the specimen, building the image point-by-point. This achievement was followed by several other laser-scanning methods, including the commonly used twophoton fluorescence microscope. Rather than using a pinhole to generate 3-D discrimination, this microscope relies on the nonlinear process of two-photon excitation to ensure that fluorescence is only generated in the focus, where the laser intensity is highest. Various advances in this field have led to improvements in resolution and contrast. Standard laboratory microscopes now regularly produce images revealing 3-D structure on the submicrometer scale. Several new methods of nanoscopy that combine optical and photophysical phenomena can even beat the diffraction limit to resolve details on the tens-of-nanometers scale.

These methods all rely on careful engineering to ensure that the optics operate at the diffraction limit, so that optimum resolution and efficiency are achieved. However, one part of the optical system—the specimen—lies outside the design specification. It is optically inhomogeneous and exhibits spatially varying refractive indices. Hence, the light focused into the specimen suffers from wavefront distortions— or phase aberrations—that degrade the resolution and imaging efficiency of the microscope. The aberrations vary from one specimen to another, so they cannot be corrected by a fixed optical design. Dynamic correction is necessary. This is where adaptive optics (AO) comes into play.

Adaptive optics was originally conceived for use in astronomical telescopes. These AO systems detect aberrations introduced by the atmosphere and use a deformable mirror to remove the aberrations before the light reaches the imaging detector. For imaging systems with small apertures, such as our eyes, the turbulence causes twinkling; for wider telescope apertures, it leads to severe image blurring that limits the resolution of the telescope.

The AO approach has been widely applied in astronomy, and it has also found application in ophthalmic imaging, laser-based fabrication, optical communications and, of course, microscopy. The adoption of AO for microscopes has brought new challenges that have required innovative solutions.
[4]

2 Aberration Measurement and Correction

For the purpose of understanding the operation of an adaptive optical system, it is best to think of aberrations in terms of distortions of an optical wavefront.

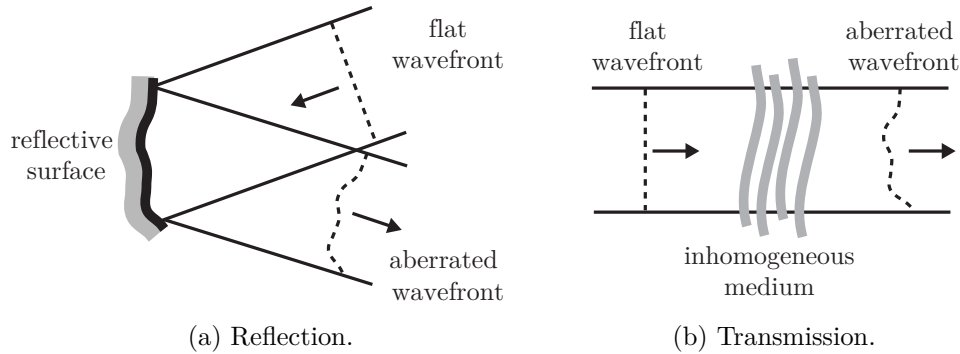(a) Reflection.   (b) Transmission.

Figure 1: Wavefront aberrations due to (a) reflection from a non planar surface and (b) caused by propagation through a non-uniform refractive index distribution. Image after [17].

Representing aberrations in this way can simplify the design, control and characterisation of adaptive optics. The choice of modes for a particular application is often influenced by some aspect of the system, such as the deformation modes of a deformable mirror or the statistics of the induced aberrations. Otherwise, the modal representation may be chosen through mathematical convenience. For example, Zernike polynomials are often used for systems with circular apertures as they form a complete, orthogonal set of functions defined over a unit circle

As with all optical systems, microscopes can also suffer from aberrations due to imperfections in the optical components. In practice, no system can be totally free from aberrations and so systems are designed to maintain aberrations below a particular tolerance for a given set of imaging conditions, such as wavelength, magnification and field of view. Significant aberrations can be introduced if a microscope is used outside its design specifications, for example at the incorrect wavelength or at a different temperature (see Chapter 11 of Ref. 9).

[3]

2.1 Direct Wavefront Sensing

2.1.1 Lateral Shearing Interferometer

2.1.2 Shack-Hartman Wavefront Sensor

2.1.3 Curvature Sensor

2.2 Indirect Wavefront Sensing

While direct wavefront sensing techniques are widely applied in Astronomy, they are less common in microscopy techniques. This for several reasons. It is not as easy to create a guiding start like point source in a biological specimen. If there are not features in the specimen that occur there naturally and which resemble a point source, one has to be implemented manually which might alter the function of the specimen or might even be toxic to the sample. Modern microscopes are also highly complex and optimized, which makes it difficult to insert a relatively large wavefront sensor. For samples with weak signal strength, it is also desirable to collect as many photons as possible for the imaging process. Splitting the beam and using a part of the light emitted from the sample for direct wavefront sensing might hence decrease the signal strength too much.

Indirect techniques do not measure the wavefront directly but instead optimize the image quality. This leads to the retrieval of the aberration and the necessary corrections. Hence these techniques don't sense the wavefront but rather improve the image quality and through this correct for wavefront aberrations. Indirect methods are used more often in industrial and medical applications. They usually

require very little additional hardware. Once the technique is optimized for a specific problem, indirect schemes are easier to implement in practice and are more prone to errors due to the lack of additional hardware (a single deformable mirror might be sufficient to implement adaptive optics in an existing microscope).

Indirect techniques include phase diversity as well as optimization of an image quality metric. Phase diversity techniques use two or more images of an extended object to make an estimation of the distorting wavefront [9]. However, this technique still requires a beam splitter, a second detector and a deformable mirror which is a significant disadvantage over image quality metric optimization where only the normally recorded image and a deformable mirror is required. It is also necessary to record images with different focus positions and hence the each phase retrieval step takes many seconds. Therefore the entire process of optimizing the wavefront takes minutes, which is to slow for most biological imaging [15]. It is for these reasons that phase diversity techniques are less common in microscopy and will not be described further. The focus of this section is therefor a general description of image quality metric techniques. Their specific properties and how they are implemented in the different microscopy techniques is then described in Section 3.

The optimization of an image quality metric is mainly a mathematical rahter then a techniquel problem. We will describe the basic principle but the derivation of the specifc metrics is beyond the scope of this paper. The interested reader will find more information on the mathematical background in reference [22, 2, 16, 6].

For these techniques, the aberration correction is performed through an iterative optimization of an image quality metric based. The metric is usually based on spatial frequencies [6] or image intensity [10]. Such optimization is either implemented empirically or by using an appropriate mathematical model. In many practical systems aberrations can be accurately represented by a small number of modes of an orthogonal basis, such as Zernikepolynomials. A sequence of images is acquired, each with a different aberration applied and the correction aberration is estimated from the information in this images. This process is repeated until the image quality is considered acceptable. The number of measurements needed to obtain an acceptable image depends strongly upon the optimization algorithm and parameters used, the mathematical representation of the aberration, and the object structure. For the earliest and most generic algorithms the number of measurements per aberration mode increases quadratically or exponentially with N, the number of corrected aberration modes [2]. The so called direct maximization method (as described in Section 3.1.1) is significantly more efficient, requiring only $N+1$ measurements for N mode. With this technique, Lukosz polynomials [16] are used to clasify the aberrations. The effects of different modes can then be separated and the optimization of each mode becomes independent and hence more efficient.

An effective model-based adaptive optics scheme should also be independent of the imaged object and should permit the separation of aberration and object influences on the measurements. This separation is also possible through the appropriate choice of optimization metric and aberration representation [6].

2.3 Aberration Correction

2.4 Deformable Mirrors

2.4.1 Liquid Crystal Spatial Light Modulators

2.5 Control Strategies

3 Adaptive Optics Methods applied in Microscopy

Adaptive optics techniques have found their way into almost all kinds of modern, high resolution microscopy techniques. These microscopes have been combined with direct wavefront sensing and sensorless AO, using deformable mirrors or spatial light modulators for aberration compensation (all of which has been described in Section 2. This includes standard widefield microscopes as well as highly sophisticated and specialized point scanning methods such as Coherent Antistokes Raman Spectroscopy (CARS) and STimulated Emission Depletion (STED) techniques. It has to be noted however, that some of these methods are themselves only a few years old. Therefor, they are still being optimized and so are the AOM techniques. It is therefore an interesting field of research with new ideas being implemented every year.

AO was first used in confocal and two-photon fluorescence microscopy, both of which are commonly used in biomedical applications. These microscopes suffer from a significant drop in signal and resolution as the focus is moved deeper into the specimen, which is caused by aberrations.[24]

AOM is also used for imaging of live specimens. Due to an increased excitation signal and improved light collection from the specimen, acquisition times can be reduced and contrast can be enhanced. Techniques that without AO are to slow for live imaging might now be usable, opening up completely new fields of research. Another advantage of AO lies in the microscopy design. Using AO methods, can help the designer to relax the aberration tolerance. This permits a significant reduction in the complexity of the optical system while maintaining diffraction limited operation.

This section will describe, using state of the art examples, how AO is implemented in both widefield and point scanning systems.

3.1 Widefield Microscopy

As mentioned above, AO techniques are being applied in widefield microscopy. In conventional microscopes, widefield illumination is provided using back light illumination or in the case of reflection or fluorescence modes, via the objective lens. The image quality depends only on the aberrations induced in the detection path and is independent of the aberrations of the illumination path. Aberration correction is therefore only necessary in the detection path and a single pass adaptive optics system will suffice [17]. Hence, the goal of AO for widefield microscopy is to restore the best possible imaging and to correct for aberrations induced both by an imperfect imaging system as well as by the imaged specimen. The latter becomes more important for thick biological samples where the light has to travel a larger distance through a medium with an inhomogeneous refractive index. This section describes how AO can be implemented in a standard transmission microscope (Section 3.1.1). Based on these early experiments, the same technique was then applied to structured light illumination (Section 3.1.2), a specialized wide field technique. Further examples include AO used in both florescence (Section 3.1.3) and multifocal multiphoton microscopy (Section 3.1.4).

3.1.1 Transmission Microscope

To implement adaptive optics with standard (incoherent) transmission microscopes, *Debarre et al.* [6] implement an indirect, sensorless and image-based adaptive optics scheme, as shown in Fig. 2. As described earlier in Section 2.2, image-based techniques do not require an additional wavefront sensor but retrieve the correction data directly form the recorded images. As with all indirect sensing schemes, the difficulty is to find a good metric for image quality, which allows to determine the appropriate correction parameters.

The presented method uses low spatial frequency content of the image as the optimization metric. The aberration is represented in terms of so called Lukosz modes. Like Zernike polynomials, the Lukosz functions are each expressed as the product of a radial polynomial and an azimuthal function. The presented technique is based on modeling the effects of aberrations on the imaging of low spatial frequencies, which Lukosz modes are are found to be ideal for.
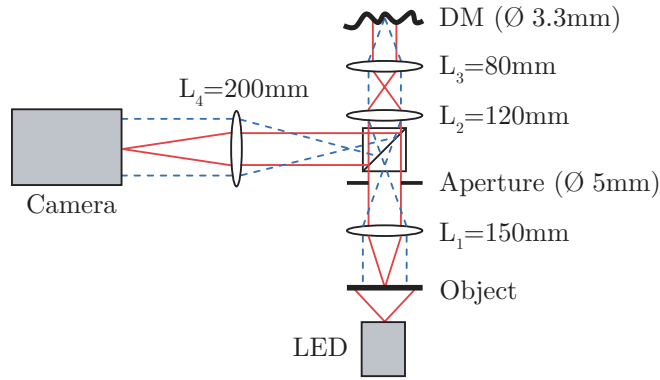
Figure 2: Schematic diagram of the experimental setup, showing a simple microscope complemented with a deformable mirror for aberration correction. Image after [6].

By modeling the aberration $\Phi(r)$ as a series of N Lukosz modes $L_i(r, \phi)$ with coefficients a_i [16]:

$$\Phi(r) = \sum_{i=4}^{N+3} a_i L_i(r, \phi), \tag{3.1}$$

they develop the optimization metric g as the sum of a range of low frequencies. It is related to the coefficients of the aberration expansion, a_i by the Lorentzian function [6]

$$g(a_i) \approx \frac{1}{q_0 + q_1 \sum_{i=4}^{N+3} a_i^2} \tag{3.2}$$

where the piston, tip and tilt modes ($i = 1, 2, 3$ respectively) have been omitted and q_0 and q_1 are both positive quantities in the frequency range of interest. The aberration correction process is then performed as the maximization of $g(a_i)$. Because of this particular aberration expansion and optimization metric, the function $g(a_i)$ shows a paraboloidal maximum that permits the use of simple maximization algorithms. Furthermore, it is shown that the optimization can be performed as a sequence of independent maximizations for each aberration coefficient.

The correction process is shown in Figure 3 for the correction of a single Lukosz mode using a scatterer specimen. Using the deformable mirror (DM), an initial aberration a_i is applied and an image is recorded. The Fourier transform and spectral density of the image are then calculated and the appropriate range of frequency components are summed, giving the metric measurements g_0. The same procedure is repeated with both negative and positive aberrations (i.e. stronger and weaker aberrations), resulting in the metric measurements g_- and g_+. Due to the paraboilc maximum of (3.2), the value of a_i that minimizes g can be calculated from as little as three measurements of $g(a_i)$.

The optimum correction aberration can then estimated by parabolic minimization as [22]:

$$a_{\text{corr}} = \frac{b(g_+ - g_-)}{2g_+ - 4g_0 + 2g_-} \tag{3.3}$$

and is then applied to the DM. To correct multiple modes, each modal coefficient is measured in the same manner before the full correction aberration containing all modes is applied. While this technique is based only on low spatial frequencies, it is shown that both low and high frequency components can be effectively corrected. In all the cases investigated, a Strehl ratio greater than 0.8, close to the diffraction limit, was obtained. This indicates that, when aberration statistics are unknown, choosing small spatial frequencies for an initial correction is a reasonable strategy. If further correction is required,they can be performed using a larger range of frequencies. *Debarre et al.* conclude that this correction scheme is largely independent of the object structure and propose that this approach also to be valid for coherent or partially coherent systems.
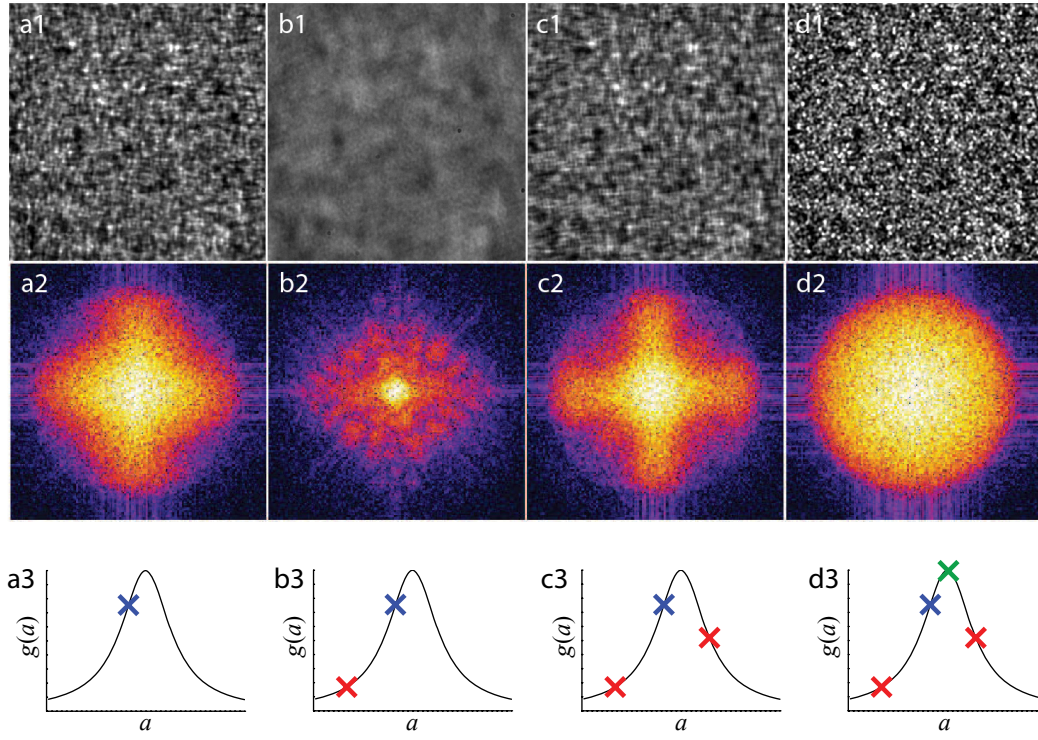
Figure 3: Correction of a single Lukosz aberration mode (astigmatism, i = 5) for a scatterer specimen and using low spatial frequencies. The first row shows the raw images of the specimen and the second row contains the corresponding spectral densities. The third row illustrates schematically the sampling of the Lorentzian curve used in the optimization calculation. (a1-a3) correspond to an arbitrary initial aberration of magnitude, (b1-b3) have an additional negative bias while (c1-c3) have an additional positive bias of equal magnitude. (d1- d3) show the corrected image calculated with the parabolic minimizationn. Image after [6].

3.1.2 Structured Illumination Microscopy

[7]

all from [6] which [7] is based on, i.e. where it is the more advandced topic

Optical sectioning microscopy is widely used to provide three-dimensional fluorescence images of biological specimens. A common way of obtaining this sectioning ability is through point scanning methods such as confocal or multiphoton microscopy [1, 2]. An alternative is to use a wide-field technique such as structured illumination (SI) microscopy, which retains the sectioning ability of confocal microscopy, but can be implemented in a conventional microscope using an incoherent light source, and without the need for scanning.

In this technique, the image of a grid is projected on the specimen so as to produce a one-dimensional sinusoidal excitation pattern in the focal plane of the objective lens. The resulting fluorescence image, consisting of both in- focus and out-of-focus fluorescence emission, is acquired by a camera. Several images are taken, each corresponding to a different grid position. As the grid pattern appears only in the focal plane, it is possible to extract an optical section from the spatially modulated component of the images via a simple calculation. [19].

In this paper we describe wavefront sensorless adaptive optics implemented in a SI microscope. It is shown that the final image quality depends predominantly on the imaging efficiency of the illumination pattern's spatial frequency. This imaging efficiency is affected much more by some aberration modes than by others. Consequently, different aberration modes can have significantly different effects on the final sectioned image.

The SI microscope relies upon the projection of a physical grid pattern into the focal plane of the

specimen. We assume that the grid object is a sinusoidal transmission mask

those aberrations that affect the grid frequency have the most significant effect on the SI microscope. It is therefore useful to separate aberration modes into two groups: those that affect the grid frequency (referred to hereon as "grid modes") and those that have no influence on this frequency ("non-grid modes"). It is clear that grid modes have a significant influence on the intensity of the sectioned image, whereas non-grid modes have comparatively little effect. The non-grid modes do however affect the resolution.

The specification of a modal aberration correction scheme requires the choice of three components: the aberration representation (the mathematical functions used to describe the aberrations), the optimisation metric (a quantity representing the image quality) and the estimator (the algorithm for estimating the correction aberration).

We would like to choose a metric function M whose maximum corresponds to the highest quality image. find an aberration expansion for which the modes act independently on the metric. This would allow the independent optimisation of each mode. When M is expressed in this form, it is clear that independent maximisation with respect to each xi is possible. Furthermore, as the function is quadratic, the maximum can be found directly from three measurements of M corresponding to three different values of xi - here they reference to [6] so maybe I should put the two together, or see this one as a more advandced version of the previous

$$M = M_0 - \sum_i x_i^2 \tag{3.4}$$

In practice, these measurements would be taken with three different trial aberrations introduced by the correction element. However, M only takes the form shown in Eq. (3.4) if the aberration representation is appropriately chosen.

for each aberration mode, the metric M was measured when adding a given amount of the considered mode, then again when substracting the same amount. Along with the measured value of M when no aberration was added, this allowed us to estimate the initial aberration present in each of the assessed modes and hence to correct these aberrations

Here we want to emphasise that the use of the appropriate metric and aberration modes means that the correction of N modes was performed using only 2N+1 measurements, thereby minimizing the increase in illumination time of the sample required for the correction.

The acquisition time per sectioned image was 300-500ms and the total time required for correction of 11 modes was 7-12s, depending on the sample.

However, M only takes the form shown in Eq. (10) if the aberration representation is appropriately chosen.

We are therefore led to the conclusion that the SI microscope, in comparison to other sectioning microscopes, is more susceptible to certain aberrations (grid modes) and more resilient to others (non-grid modes).

Whilst the SI microscope relies upon a relatively simple optical principle, the image formation process has a complex mathematical description. Similarly, the derivation of a modelbased, sensorless, adaptive optical scheme is a complex process. However, our results show that the scheme is effective in correcting specimen-induced and system aberrations and restoring image quality. For the samples presented here, we found that the aberration mainly consisted of astigmatism, coma and spherical aberration modes.

The adaptive scheme described here has significant advantages over model-free algorithms in that the aberration correction can be estimated using a small number of measurements (2N+1 for N aberration modes). Moreover, as the scheme is mostly independent of the object structure, the appropriate modes have only to be determined once and the same scheme can be used for any specimen.We have also shown that aberration correction can be effectively combined with background subtraction to further improve SI microscope images. In the results presented here, aberration correction was performed as an average over an image frame and therefore would not correct for any local variations in aberrations. If these variations were found to be significant, the image could be formed from several sub-images for which independent aberration correction would be performed.
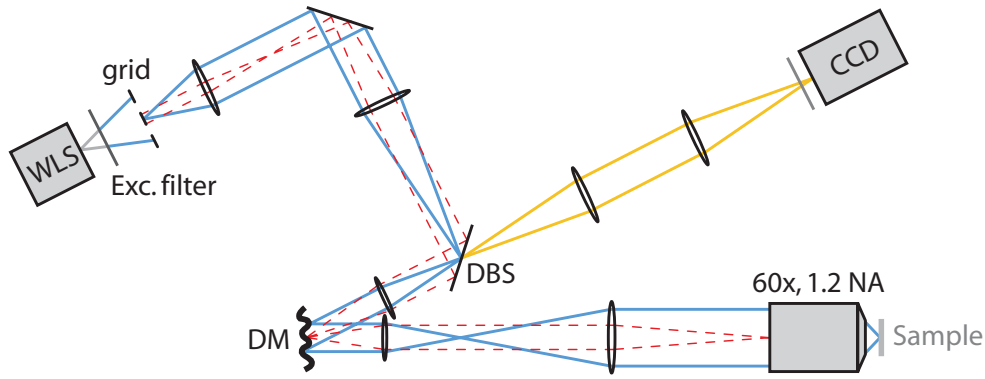
Figure 4: Experimental setup for structured illumination microscopy with aberration correction. WLS - white light source, DM - deformable mirror, DBS - dichroic beamsplitter. The blue rays mark the illumination path; the detection path is shown in yellow. Image after [7] .

We have presented a general method that provides an optimal aberration expansion for a chosen optimisation metric. This relied upon the derivation of an inner product from a mathematical model of the imaging process, followed by an orthogonalisation process applied to a set of basis functions, such as the Zernike functions. This process reveals a wealth of information about the effects of different aberration modes on an imaging system – for the SI microscope, it enabled us to derive the sets of grid modes and non- grid modes. **This method could equally be applied to any sectioning microscope to derive aberration expansions that are best suited to that application.**

(a) Uncorrected.   (b) Corrected.   (c) Line Scan.
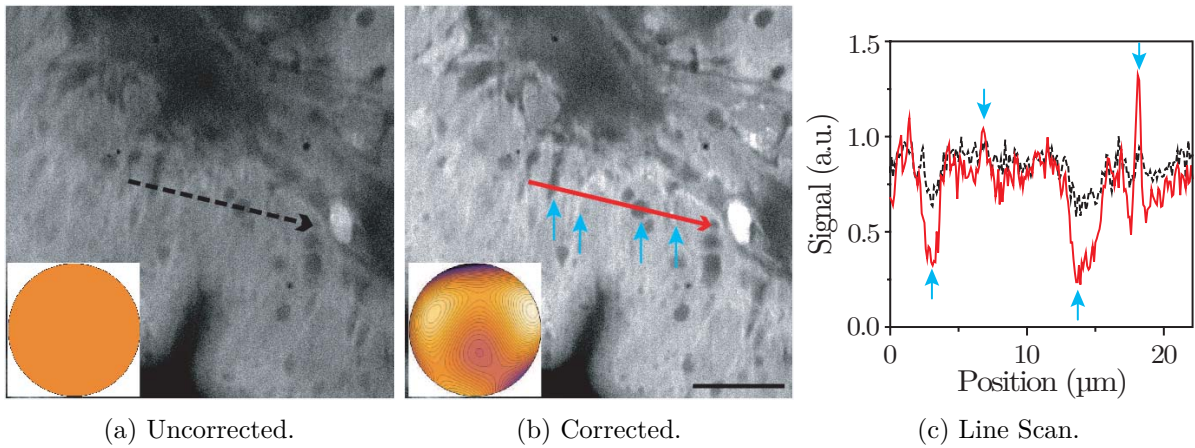
Figure 5: Aberration correction in structured illumination microscopy. A fluorescent mouse intestine sample was imaged (a) without (b) with aberration correction with inserts showing the phase induced by the deformable mirror. (c) Profile along the lines drawn on the images, both profiles normalized so that their mean value is identical. As a result of the resolution improvement, the contrast of small sample features (blue arrows) are better defined after (red solid line) rather than before (black dotted line) correction. The imaging depth was approximately $10\,\mu$, sacle bar size $10\,\mu$. Image after [7] .

3.1.3 Fluorescence Microscopy

next section, all from [14] uses a standard widefield flourescene microscope but use AOM to correct for spherical aberration due to depth -> no specimen induce correction uses deconvolution to get out of foucs photons corrected

In this paper, we concentrate on the depth dependent aberration which can quickly become serious. Imaging 20 mum a live sample (index of refraction 1.36) with an oil immersion lens causes the peak intensity of the point spread function (PSF) to drop 3-fold and the width of the PSF in the axial

direction to increase by 2-folds. [14]

Because wide-field microscopy captures as efficiently as possible every emitted photon ultimately minimizing the sample excitation dose, it is well suited to in vivo imaging in sampleswhere scattering is not too large. Although the out-of-focus photons are in thewrong place, they can be effectively re-assigned to the location of emission by constrained deconvolution algorithms [25]

The problem of depth aberrations can be solved by matching the sample index and the index of the immersion medium, but this is frequently not feasible or desirable. For example, the index of fixed cells can be matched to that of the immersion oil, but this option is not available for live imaging.

An important drawback to most schemes that have been proposed so far is that they require several images to be taken to optimize the aberration correction. This presents a serious problem for live imaging in biology because the fluorescence intensities can be weak and susceptible to rapid bleaching.

The approach we follow is to correct the depth aberrations withanopen-loop predictive algorithm similar totheapproach taken by Potsaid et al. in correcting off-axis aberrations. This is possible because the depth aberration can be calculated for a given depth into the sample. The depth aberration is the result of depth-dependent path length differences.

Correcting depth aberrations with a DM improves both the peak intensities and the deconvolution of images taken below the cover slip by removing the depth aberration. This allows the use of fast space-invariant deconvolution algorithms instead of depth-dependent algorithms. This is significant because it improves both the signal-to-noise ratio and the resolution in biological imaging where photons are in short supply. Unfortunately, the performance does not yet achieve what is theoretically possible.
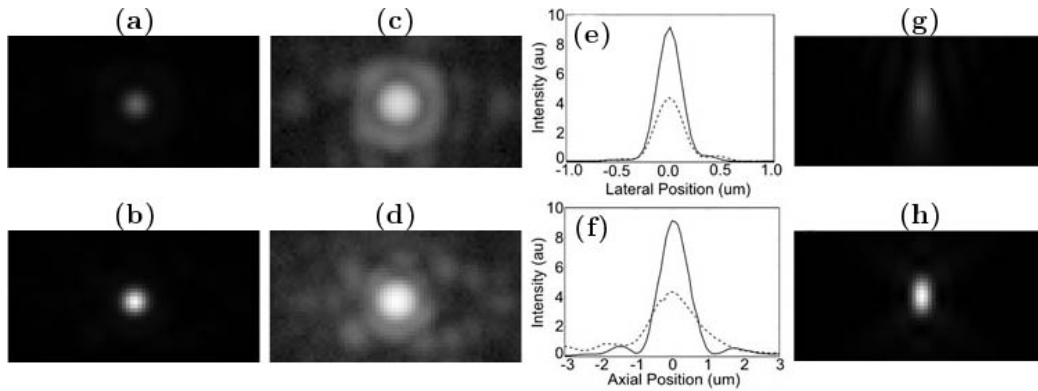

Figure 6: Images of a 200 nm bead 67 μm below the cover slip in a water/glycerol mixture with n = 1.42. (a) Uncorrected image of in-focus plane. (b) Corrected image of in-focus plane. Images (c) and (d) are the same as (a) and (b), respectively, but on a logarithmic scale for better visualization. (e) and (f) are line profiles of the intensity through the center of the bead along the lateral and the longitudinal axis, respectively. The dashed line is from the uncorrected image and the solid line is from the corrected image. (g) and (h) are simulations of the PSF. Images based on [14].

The first is the effect of uncorrected aberrations from the sample and the optical path, which decrease the maximum intensity at the cover slip, but in a way that does not add linearly to the depth aberration. Thus only a fraction of the dispersed photons can be restored to the central peak. In closed loop AO systems, system aberrations are automatically compensated at each position (Wright et al., 2007), but in an open-loop system this is not possible. The second factor is the inability of the mirror to precisely conform to the shape given by Eq. (1). The residual error of the mirror shape increases with depth (see Fig. 3c) so that as the imaging plane goes deeper and the possibility for improvement becomes greater, the improvement in peak intensities decreases

Lastly, the ultimate goal of applying adaptive optics in microscopy is to correct all aberrations including those introduced by the refractive index variations of the sample itself. [14]

3.1.4 Multifocal Multiphoton Microscopy

[1]

3.2 Point Scanning Microscopes

Scanning optical microscopes are widely used for high resolution imaging, mainly because certain implementations provide three-dimensional resolution with optical sectioning and are thus particularly useful for imaging the volume structures of biological specimensz. In these microscopes, illumination is provided by a laser that is focused by an objective lens into the specimen. The light emitted from the specimen is collected, usually through the same objective lens, and its intensity is measured by a single photodetector. The focal spot is scanned through the specimen in a raster pattern and the image is acquired in a point-by-point fashion. The resulting data are stored and rendered as images in a computer.

Several other point scanning microscope modalities have been introduced, including two-photon excitation uorescence (TPEF) microscopy, second harmonic generation (SHG) and third harmonic generation (THG) microscopy, and coherent anti-Stokes Raman (CARS) microscopy.
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3.2.1 Confocal Microscopes

The most common example of this type is the confocal microscope, which can be operated in reflection or fluorescence mode. Three-dimensional resolution is achieved by the placement of a pinhole in front of the photodetector. In a reflection mode confocal microscope, the illumination is scattered by objects not only in the focal region, but throughout the focusing cone. In fluorescence mode, emission is generated in the focus but also in out-of- focus regions. The pinhole ensures that mainly light from the focal region falls upon the detector and light from out-of-focus planes is obscured. It is critical in the confocal microscope that both the illumination and detection paths are diffraction limited. This ensures that i) the illuminating focal spot is as small as possible, and ii) that the focus is perfectly imaged on to the detector pinhole. Therefore, in an adaptive confocal microscope, aberration correction must be included in both paths. This dual pass adaptive system can usually be implemented using a single deformable mirror, if the path length aberrations are the same for both the illumination and the emission light. This is the case if there is no significant dispersion in the specimen or chromatic aberration in the optics.

A pinhole is not required to obtain three-dimensional resolution, so most TPEF microscopes use large area detectors to maximise signal collection. Although they rely upon other physical processes, non-linear imaging modalities such as SHG, THG and CARS exhibit similar resolution properties. When using large area detectors, the fidelity of imaging in the detection path is unimportant so the effects of any aberrations in this path are negated. It follows that single pass adaptive optics is appropriate for these microscopes as aberration correction need only be implemented in the illumination path.

Adaptive optics systems have been successfully combined with several point- scanning microscope systems including confocal,13 TPEF,6, 14, 15 harmonic generation,16, 17 CARS.18 Example images of aberration correction in an adaptive THG microscope are shown in Fig. 10.

[21] [5]

3.2.2 Two-Photon Fluorescence Microscopy

[23] [8] [18]

3.2.3 Harmonic Generation

[13] [20]

3.2.4 CARS

[26]

3.2.5 STED

[11]

4 Future Prospects

More work must be done before AO can become a regular component of laboratory microscopes. Most AO microscopes are too complex to set up, and their application can be limited by the robustness of operation. The development of automated alignment and calibration procedures would enable the turnkey operation needed to make these systems more practical.

The effectiveness of AO microscopy is mostly compromised by aberration measurement, rather than by currently available correction devices. More sophisticated wavefront sensors or sensorless optimization schemes will extend the microscope's ability to cope with large and more complex aberrations. An obvious goal is to develop "realtime" aberration sensing to increase the speed of correction. Coupled to this is the desire to reduce the exposure of specimens during the measurement process—an essential step when using microscopes for live imaging.

Aberrations can change significantly across a single field of view because the refractive index of the specimen varies throughout its volume. So far, the methods used in adaptive microscopes have provided only a fixed aberration correction for each image. This is sufficient if the imaged region is small enough that aberrations do not vary significantly across the field.

One way to overcome this limitation would be to apply multiconjugate AO to microscopes. This method has been applied in astronomy using multiple deformable mirrors to compensate for multiple aberrating layers in the atmosphere. A similar approach in microscopy would compensate for the 3-D refractive index distribution, although the optical system would become considerably more complex.

Further advances in AO will extend the capabilities of high-resolution microscopes to reveal functional and structural information from deep within biological tissue. Currently, optimum performance is often limited to thin regions near to the coverslip, sufficient for imaging individual cells, but of rather limited practicality for tissue imaging. AO promises to help move microscopy into a new regime in which biological studies that were previously confined to cell cultures can be performed in thick tissue and even in live specimens.

Add short part about STORM/PALM which are not using adaptive optics yet... [12]

5 Conclusion

abberations that are important for imaging system differ a lot, some need correction of both illu and imaging, some use direct, other indirect methods, some only need correction of some abberation modes, others need as much as possible...important to choose correct system for given problem and figure it our in detail before ordering something

AO is applicable to almost all microscopy techniques...

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