

Optical Imaging in Biology and Medicine
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Adaptive Optics for Microscopy

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Abstract

Images with better resolution have always been a challenging topic and an important goal in many fields of science. Specially, in biophotonics. Although new techniques of superresolution are appearing and being developed, in any optical system there are always aberrations that often compromise the resolution and brightness of images. Thus, to overcome this problem it is used Adaptive Optics (AO), which is a relatively new field in biophotonics imaging. In this paper we have reviewed the basic principles of AO, focusing our interest in how we obtain the aberration information (i.e., direct and indirect sensing) and particularly, how AO is applied in the main microscopy techniques (i.e., widefield and point scanning techniques). We have shown that in almost all techniques AO have been used. However, the increase in resolution depend on many factors such as the specimen under study and the microscopy technique used.

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1 Introduction

It is well known that optical aberrations degrade the resolution and brightness of images. That means a reduction in both lateral and axial resolution and a fall in signal intensity. Aberrations, in general terms, can be defined as the wavefront distortions with respect to ideal spheres, these distortions are due to imperfections in any part of the optical system. In microscopy, aberrations may arise from the microscope itself or the specimen under study [7]. Therefore, aberrations always limit in some way the final image quality and vary from one specimen to another, so they cannot be corrected by a fixed optical design. Dynamic correction is necessary.

That is the reason why scientists have been trying to overcome this problem for many years. The best approach to it is what nowadays is called Adaptive Optics (AO). The first proposal of the use of AO technology was suggested in the year 1953 in the context of astronomical optics for the compensation of the aberrating effects of the atmosphere [4].

The main idea of the AO is the modulation of an incoming wavefront in such a way that we can record an image without aberrations. It is based upon the principle of phase conjugation: the correction element introduces an equal but opposite phase aberration to that present in the optical system. In order to do that, we need to be able to measure these distortions reliably. The most direct way is to use a wavefront sensor, the Shack-Hartmann [37, 30] or Curvature Sensors [33] are examples of it. Also, interferometric techniques have been used to measure aberrations [39]. Nevertheless, there are indirect methods in which aberrations are estimated using an algorithm and do not employ a wavefront sensor, they are called sensorless techniques [8]. In addition to the wavefront sensing, we need the adaptive element to modulate the aberrations before the light reaches the imaging detector, this is usually a deformable mirror or a liquid crystal spatial light modulator (LC-SLM). Finally, we need a control system that processes the aberrations information and uses it to monitor the adaptive correction element.

Although Adaptive Optics systems have been introduced in applications such as astronomy, laser beam shaping, optical communications, data storage and ophthalmology [9], when it is applied to microscopy is not always trivial and it requires a different approach than in the other fields. One particularly difficult thing in AO microscopy is how the aberration information is obtained in each of the different microscopy techniques.

The optical microscope techniques can be divided in two main groups: the widefield techniques and the point scanning techniques. Some examples of the first group are the conventional transmission microscopy, the structured illumination microscopy and the fluorescence microscopy. Some examples within the second group are the confocal microscopy, Stimulated Emission Depletion (STED) or the non-linear microscopy such as Two-Photon Excitation Fluorescence (TPEF), Second Harmonic Generation (SHG), Third Harmonic Generation (THG), Coherent anti-Stokes Raman (CARS).

In this report, in order to explain how adaptive optics and microscopy link together, we will start explaining the basis of adaptive optics. That means a brief review of the concept of aberrations and how they are most commonly characterized. After that, we will explain in more detail the main methods for wavefront sensing, the main aberration corrector devices and some control strategies. Finally, we will show some applications of the AO in different widefield and point scanning microscopy techniques. The last part will be a short explanation of future prospects and conclusions.

2 Aberration Measurement and Correction

In practice, no optical system can be totally free from aberrations. That means that all the rays coming from the same object point and going through an optical system will not converge into the same point at the image plane. In other words, the wavefront is distorted with respect to an ideal one when passing through a real system. Thus, we can define the Wavefront Aberration Function as the optical path difference between the aberrated (real) wavefront and the reference (perfect) wavefront. There are some ways to characterize mathematically the aberrations. In systems with circular symmetry (circular apertures) it is very common to use the Zernike polynomials (Eq. (2.1)) because they form a complete, orthogonal set of functions defined over a unit circle. This property gives independence between these

functions. That implies that it is possible to associate to each polynomial a specific an independent weight that will contribute to the description of the Wavefront Aberration Function.

$$W(\rho, \phi) = \sum_n^k \sum_{m=-n}^{m=n} c_n^m Z_n^m(\rho, \phi), \quad (2.1)$$

Where $W(\rho, \phi)$ is the Wavefront Aberration Function in polar coordinates at the exit pupil, c_n^m are the Zernike coefficients and $Z_n^m(\rho, \phi)$ are the Zernike modes (or polynomials). As we can see in the equation, the Wavefront Aberration Function is a linear combination of polynomials. Therefore, the more polynomials (i.e. modes, $Z_n^m(\rho, \phi)$) we get the better characterization of the $W(\rho, \phi)$ function we have. Representing aberrations in this way can simplify the design, control and characterization of the Adaptive Optics system. In general, we can obtain an aberrated wavefront when it is reflected to a non-planar surface or when it is going through an inhomogeneous media (Fig. 1).

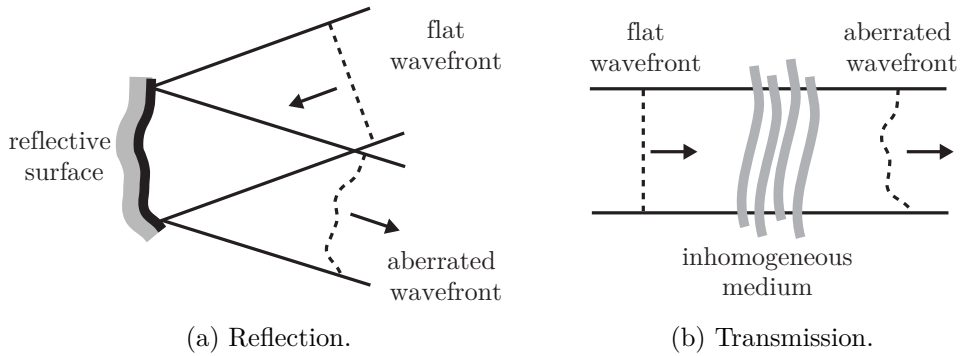


Figure 1: Wavefront aberrations due to (a) reflection from a non planar surface and (b) caused by propagation through a non-uniform refractive index distribution [7].

In biological microscopy, the two potential sources of aberrations are the optics and the specimen under study. Regarding the optics, one important parameter that has to be taken into account is the Numerical Aperture (NA), aberrations become more significant for those microscopes employing higher NA. Regarding the specimen, aberrations comes basically from the variations in refractive index due to the three-dimensional nature of cells and tissue structures. In general, they become greater in magnitude as long as we focus deeper. Also aberrations can be produced by the difference of refractive index between the microscope coverslip and the specimen mounting medium [7]. Although it is known where the aberrations come from, it is not always easy to measure them and implement the measure inside the optical system. There are different classifications of Wavefront Sensing in the bibliography [38]. Here we will use the one of Martin J. Booth [7] and we will explain the most common methods of wavefront sensing applied to microscopy.

2.1 Direct Wavefront Sensing

Direct Wavefront Sensing is based on a direct measure of the phase gradient or the wavefront slope, it is considered as an aperture-plane sensing. Within this group there are several techniques such as interferometric, although in general the most used is the Shack-Hartmann.

This last technique is based on a two-dimensional array of a few lenslets -a matrix of microlens-, all with the same diameter and the same focal length, that allows us to measure the wavefront's slope at the exit of the optical system. In other words, it allows us to reconstruct the wavefront aberration function from the local slope's changes of an aberrated wavefront in relation with a reference wavefront. That is achieved measuring the displacements $(\Delta x, \Delta y)$ from each image given by each microlens (Fig. 2).

We start from an aberrated beam going through the matrix of microlens before it is detected in a recorder device. The beam is then spatially sampled into many individual beams (one for each microlens) by the lenslet matrix and forms multiple spots in the focal plane of the lenslets. Finally, the

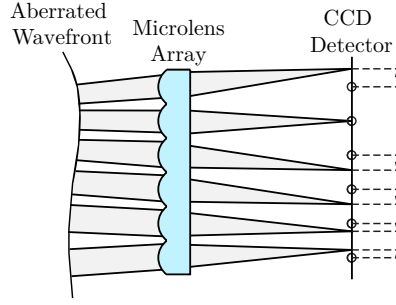


Figure 2: Two-dimensional section of Shack-Hartmann matrix of microlens. When an incoming wavefront goes through the matrix is divided in multiple beams. In the focal plane of the matrix we have multiple spots. If the wavefront is not aberrated, each spot will be placed along the axis of each microlens. If it is aberrated, it will be displaced with respect to this axis. Image after [25].

recorder device -that is placed at the focal plane of the matrix- registers multiple spots. This device is usually a CCD camera. The typical lenslet diameters range from about 100 to 600 μm and the typical focal lengths range from a few millimeters to about 30 mm [31].

We must note that direct sensing needs a well defined wavefront in the pupil of the system or in other words, a point-like emitter. In this situation might not happen in biological microscopes where we are studying three-dimensional specimens, because the light emitted by the specimen may come not only from the focal point. This fact brings us to a couple of problematic situations. The first one is the superposition of wavefronts in the pupil which its effects will depend on the coherence of the emitted light. If it is coherent we will have interference in the pupil, thus we will have ambiguous sensor readings and this kind of sensing will not be suitable for aberration correction. In this sense, if in the focal region the specimen behaves as a point-like scatterer (it will emit incoherent light) the sensor will be able to measure the aberrations produced in the emission path, which in principle they should be the same as in the illumination path. But, if our specimen acts as a planar mirror-like in the focal region, the sensor will just be able to measure twice the even components of the aberrations produced in the illumination path (or emission path), that is because the "mirror behavior" will spatially invert the aberrations in the illumination path (Fig. 3). Let us analyze it in detail. We know that the measured aberration ($W(\rho, \phi)$) is the sum of the illumination and emission path aberrations. Also we can denote the measured aberration as the sum of its even and odd components. All together become,

$$W(\rho, \phi) = W(\rho, \phi)_i^{\text{even}} + W(\rho, \phi)_i^{\text{odd}} + W(\rho, \phi)_e^{\text{even}} + W(\rho, \phi)_e^{\text{odd}}, \quad (2.2)$$

Due to the spatial inversion we know that $W(\rho, \phi)_i^{\text{even}} = W(\rho, \phi)_e^{\text{even}}$ and $W(\rho, \phi)_i^{\text{odd}} = -W(\rho, \phi)_e^{\text{odd}}$, therefore the measured aberration can be simplified as,

$$W(\rho, \phi) = 2W(\rho, \phi)_e \quad (2.3)$$

We have shown that in planar mirror-like structures we lose information about aberrations. Thus, in this cases it is not a good option to implement a direct sensing. Regarding microscopy techniques, we must note here that fluorescence emission is an incoherent process, but the non-linear processes generate a coherent signal.

The other problematic situation that we can have in direct sensing due to three-dimensional nature of the specimen is that our sensor might detect more intensity signal from the light scattered out-of-focus than the scattered in the focal region. In order to overcome this problem it can be used a spatial filter between objective and sensor. This filter performs in a similar way than the pinhole used in confocal microscopy. Also it is possible to use coherence gating to exclude out-of-focus light instead of the spatial filter, although it is a more complex method. We must recall here that the light emitted in the

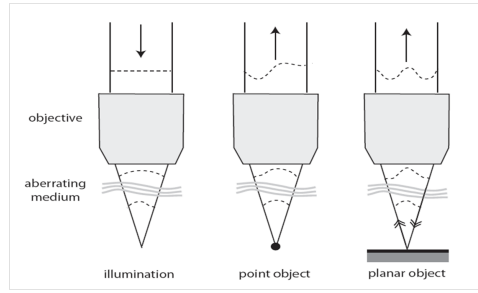


Figure 3: Representation of the two effects due to the specimen structure on wavefront measurements. The left figure shows how the wavefront is aberrated in the illumination path. In the center it is shown a point-like scatterer. Only the emission path is measured. In the right figure it is shown a planar reflector. The illumination wavefront is spatially inverted on reflection before acquiring further aberration in the detection path [7].

non-linear processes is confined mostly in the focal region.

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 [15]. 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 too slow for most biological imaging [22]. 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 therefore 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 rather than a technical problem. We will describe the basic principle but the derivation of the specific metrics is beyond the scope of this paper. The interested reader will find more information on the mathematical background in reference [32, 6, 23, 12].

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 [12] or image inten-

sity [17]. 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 Zernike polynomials. 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 [6]. 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 [23] are used to classify 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 [12].

2.3 Aberration Correction

The wavefront correctors are the essential device of an adaptive optics system. Their aim is to provide a certain phase profile of the incident wavefront by changing the physical length over which the wavefront propagates or the refractive index of the medium through which the wavefront passes. They are based on mirror technology (Fig. 4) or on liquid crystal technology. The first ones change the phase by adjusting their surface shape (i.e., change their physical length while keeping the refractive index constant) and the second ones keep the physical length constant and rely on localized changes in refractive index. The mirror-based correctors are wavelength and polarization independent and can be reconfigured at rates of a few kilohertz [7]. They can have a continuous surface (i.e., discrete actuator, bimorph or membrane mirrors) or segmented surface (i.e. piston-only or piston/tilt mirrors). Unlike continuous mirrors, the segmented mirrors have gaps between the segments that reduce the efficiency and quality of the correction, although they can achieve much better wavefront fitting.

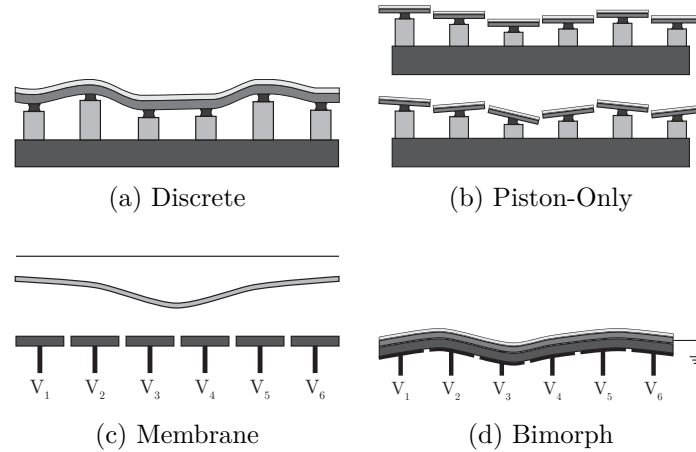


Figure 4: The four main mirror correctors. (a) Discrete actuator deformable mirrors consist of a continuous, reflective surface and an array of actuators, each capable of producing a local deformation in the surface. (b) Piston-only segmented correctors consist of an array of small planar mirrors whose axial motion (piston) is independently controlled. Piston/tip/tilt-segmented correctors add independent tip and tilt motion to the piston-only correctors. (c) Membrane mirrors and (d) Bimorph mirrors. Image after [31].

Regarding the liquid crystal-based mirrors, they change the refractive index electronically or optically, are wavelength and polarization dependent and can reach velocities of just a few tens of hertz. The

nematic liquid crystal is the most common for AO applications. In general, they are much cheaper than the mirror-based correctors, are capable of producing more complex phase patterns but they have lower light efficiency.

In AO microscopes the first choice in most cases is the deformable mirror because of their high detection efficiency. Besides, they are better suited for fluorescence techniques due to their polarization independent behavior. However, in particular cases, LC-SLM can be enough if aberration correction is only needed in the illumination path.

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. Therefore, 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 [35].

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 too 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 [24]. 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.

Many other highly specialized widefield microscopy techniques have been developed and for most of those, AO schemes for aberration correction and resolution optimization have been presented. Two widefield microscopy techniques will be presented in this section. First the implementation of AO in a standard transmission microscope (Section 3.1.1) using a sensorless wavefront sensing scheme is explained. How the theoretical background of this technique can be applied to more sophisticated microscopy schemes is then shown on the example of structured light illumination (Section 3.1.2), a specialized wide field technique.

Not covered by this report is the application of AO using a direct wavefront sensing scheme as presented by *Azucena et al.* in 2011 using a Shack–Hartmann wavefront sensor, a fluorescent reference source, and a deformable mirror [3]. Adaptive optics can also be used to correct for aberrations in fluorescence microscopy. There, the aberration caused by a refractive index mismatch between

sample, cover plate and immersion medium can be calculated theoretically and is then corrected [21] or the aberration is measured using a guide-star technique [2] as described in Section 2. AO is also applicable in multifocal multiphoton microscopy [5, 11].

3.1.1 Transmission Microscope

To implement adaptive optics with standard (incoherent) transmission microscopes, *Debarre et al.* [12] implement an indirect, sensorless and image-based adaptive optics scheme, as shown in Fig. 5. As described earlier in Section 2.2, image-based techniques do not require an additional wavefront sensor but retrieve the correction data directly from 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.

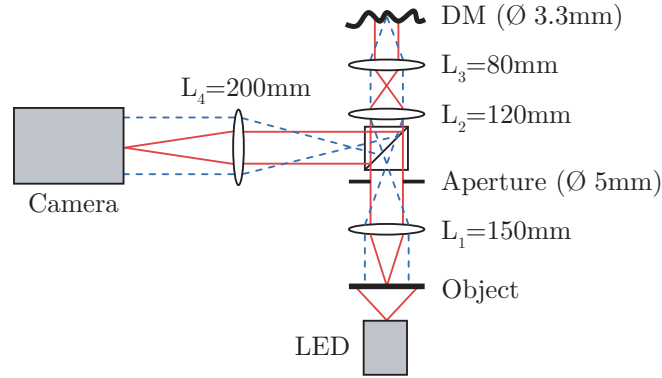


Figure 5: Schematic diagram of the experimental setup, showing a simple microscope complemented with a deformable mirror for aberration correction. Image after [12].

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 found to be ideal for.

By modeling the aberration $\Phi(r)$ as a series of N Lukosz modes $L_i(r, \phi)$ with coefficients a_i [23]:

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

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 [12]

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

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 6 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

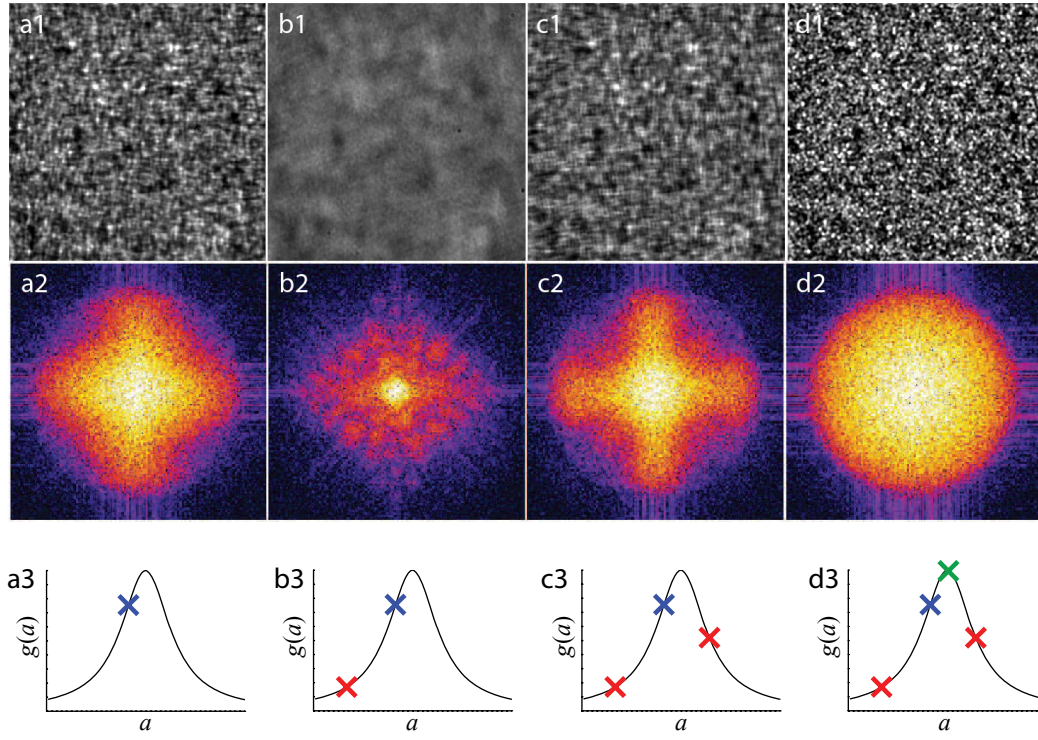


Figure 6: Correction of a single Lukosz aberration mode (astigmatism, $i = 5$) for a scatterer specimen and using low spatial frequencies. The first row shows the raw images of the specimen and the second row contains the corresponding spectral densities. The third row illustrates schematically the sampling 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 minimization. Image after [12].

aberrations), resulting in the metric measurements g_- and g_+ . Due to the parabolic maximum of Eq. (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 be estimated by parabolic minimization as [32]:

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

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.

3.1.2 Structured Illumination Microscopy

It is often desired for biological samples to produce clear images of focal planes deep within a thick sample (i.e. optical sectioning) and common techniques include point-scanning techniques such as confocal or multiphoton techniques which are described in Section 3.2.

Widefield techniques such as Structured Illumination (SI) microscopy can also provide optical sec-

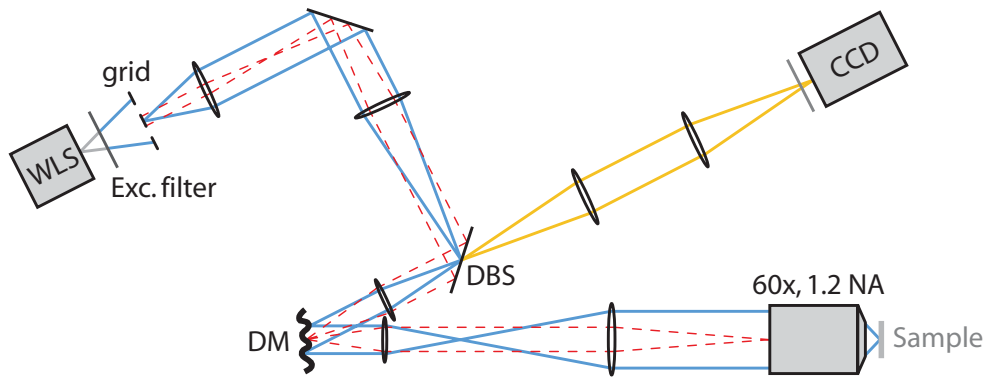


Figure 7: Experimental setup for structured illumination microscopy with aberration correction. WLS - white light source, DM - deformable mirror, DBS - dichroic beamsplitter. The blue rays mark the illumination path; the detection path is shown in yellow. Image after [13] .

tioning. However, there the sectioning is realized using a standard microscopes, an incoherent light source and without the need for a scanning mechanism. For SI microscopy, a grid is imaged into the specimen to produce a one-dimensional sinusoidal excitation pattern in the focal plane. The resulting sinusoidal fluorescence image, consisting of both in- focus and out-of-focus fluorescence emission, is then normally recorded. Several images are taken, each corresponding to a different grid position equivalent to three different phase shifts of the grating. The grid pattern only appears in the focal plane while it is blurred in the out of focus regions. Hence, it is possible to extract an optical section from the spatially modulated component of the images via a simple calculation.

Based on the SI microscopy technique presented by *Neil et al.* in 2005 [27] as well as their earlier work on indirect wavefront sensing using a conventional microscope [12] in 2007 (described in the previous section) *Debarre et al.* combined both techniques in 2008 and proposed an AO scheme for use in SI microscopy [13].

They again present a sensorless wavefront detection scheme, which is shown in Fig. 7. The method to obtain the aberration correction is similar to the one presented and explained in the previous section. The authors derive an inner product from a mathematical model of the imaging process, followed by an orthogonalization process applied to a set of Zernike polynomials. Based on that, a general method providing an optimal aberration expansion for the chosen optimization metric is presented. This process yields information about the effects of different aberration modes in of the SI microscope. *Debarre et al.* show that the image quality mainly depends on the imaging efficiency spatial frequency of the illumination pattern. This imaging efficiency is affected much more by some aberration modes (called grid modes) than by others (called non-grid modes) . 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 results of the implemented AO scheme is shown in Fig. 8 for aberration correction on a fluorescent mouse intestine. The image contrast and sharpness improvement is clearly visible in image 8b compared to the uncorrected image in 8a. As a result of the aberration correction, and as shown in Fig. 8c, the contrast of small sample features (blue arrows) is better defined after (red solid line) rather than before (black dotted line) correction.

The authors also explain an additional benefit of aberration correction for structured illumination microscopy. That the adaptive element can also be used to improve the rejection of the out-of-focus fluorescence. When imaging thick specimen, noise fluctuations in the fluorescence signal between the three successive widefield images obtained for the maximization process result in a large out-of-focus background in the calculated sectioned images. Since this background arises from fluorescence generated outside the focal plane, it is not sensitive to the presence of aberrations. By applying large aberrations in a number of grid modes, the grid pattern is suppressed and only the out-of-focus noise can be measured. By subtracting this aberrated image from the original sectioned image, the fluorescent background can be efficiently removed, leading to greatly improved contrast of the in-focus structures.

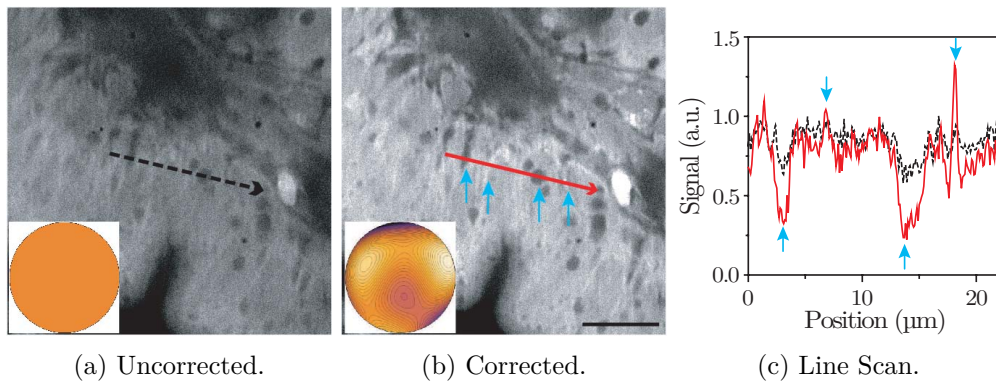


Figure 8: Aberration correction in structured illumination microscopy. A fluorescent mouse intestine sample was imaged (a) without (b) with aberration correction with inserts showing the phase induced by the deformable mirror. (c) Profile along the lines drawn on the images, both profiles normalized so that their mean value is identical. As a result of the resolution improvement, the contrast of small sample features (blue arrows) is better defined after (red solid line) rather than before (black dotted line) correction. The imaging depth was approximately $10\text{ }\mu\text{m}$, scale bar size $10\text{ }\mu\text{m}$. Image after [13] .

The aberrations can also change significantly with depth and hence using the same correction for different depths can result in a degradation of the image quality. The correction can however be adapted for different imaging depths in the sample. This permits improvement of the image quality throughout an axially extended sample. It is furthermore possible to determine the appropriate modes once and use the same scheme for any specimen, as the scheme is mostly independent of the object structure. Alternatively, if one wants to correct for local variations in aberrations the image could be formed from several sub-images for which independent aberration correction would be performed.

In conclusion, the authors present a sophisticated, easy to implement and highly versatile AOM scheme which allows for aberration correction induced by the optical system, the specimen or the focus depth. While the presented scheme uses a widefield microscope, *Debarre et al.* are also optimistic that similar AO methods based on indirect, image based aberration detection can be applied to point-scanning methods.

3.2 Point Scanning Microscopes

Just as with the widefield techniques, adaptive optics quickly found its way into point scanning techniques to improve the image and signal quality.

Scanning methods are useful for imaging biological specimens, since they can provide high resolution imaging in three-dimensions. Illumination is usually provided by a laser that is focused into the sample. The light emitted or reflected from the specimen is collected, usually through the same objective lens, and its intensity is measured by a detector. Since this only provides information about the intensity at a single spot, focal point is then scanned through the specimen and point-by-point the image is acquired.

Several other point scanning microscope modalities have been introduced, including Two-Photon Excitation Fluorescence (TPEF) microscopy, second harmonic generation (SHG) and third harmonic generation (THG) microscopy, and coherent anti-Stokes Raman (CARS) microscopy.

- using a fluorescence microscope, the smaller FWHM, provided by the optimized DMM, will increase the excitation intensity leading to a higher fluorescent signal for the same laser beam input power

- [20, 29] -> Both the second- and third-harmonic intensity signals are used as the optimization metric
- Aberration correction is performed to compensate both system- and specimen-induced aberrations by using an efficient optimization routine based upon Zernike polynomial modes - images of live mouse embryos show an improved signal level and resolution.
- The peak intensity increased by almost 50 % and the FWHM decreased by 14 % to $1.22\text{ }\mu\text{m}$ compared with a value of $1.15\text{ }\mu\text{m}$ for an unaberrated

system

[40] - signal improvement 3x for samples at a depth of 700 μm and 6x for muscle tissue at a depth of 260 μm - completely random optimization, approach is well suited to CARS microscopy, since photo-bleaching does not occur - optimization algorithm typically converged after 3000 mirror shapes, mirror speed up to 1kHz this can be pretty fast

[18] (2012) - STED principle [19] (1994) - STED phase mask as well as aberration correction realized using the SLM - aberration correction in both depletion beam (better resolution) and in the excitation beam path (better signal and less noise) - due to depletion image brightness as metric not good, introduced a new metric that seeks to optimize both image brightness and image sharpness in a combined approach, - imaging through a #1.5 coverglass (0.15 mm thicknes) and 55 μm glycerol, Comparing the axial profiles of the STED and AO STED reveals a 5-fold increase in the peak signal as well as a 3.2-fold improvement in resolution

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3.2.1 Confocal Microscopes

The confocal microscopy can be operated in reflection or fluorescence mode. Both are a dual pass system, which means that either the illumination path and the emission path have to be corrected in an AO confocal microscope.

The first attempt to apply AO in confocal microscopy was done by *Martin J. Booth et al.* [10]. They implemented indirect wavefront sensing to a confocal fluorescence microscope in a closed-loop way. Aberration measurement and correction was done sequentially. First a preset positive bias aberration was introduced by a deformable membrane mirror. An image scan was taken and all of its pixel values were summed and averaged to give the value of W_1 . Then, it was added to the system the equivalent negative bias aberration, obtaining W_2 . They had shown before that the value of the difference signal, $W = W_1 - W_2$, is approximately proportional to the amount of the Zernike mode Z_i present in the sample. They applied this procedure for several different Zernike modes, updating the mirror shape each time. A simple representation of the experimental set-up is shown in Fig. 9.

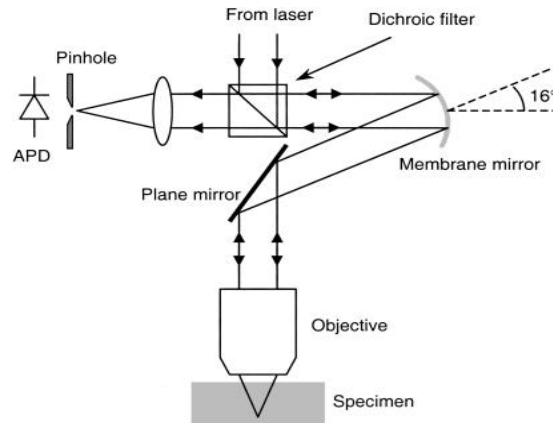


Figure 9: The illumination beam was passed through a beam expander and reflected by the membrane mirror such that the angle between the incident and reflected beams was 16°. Then it passed into the objective lens focusing the light into the specimen. Fluorescence light from the specimen was collected by the same objective. In this configuration, the membrane mirror can compensate for aberrations introduced into both the illumination and emission optical paths.

With this sequential method for correcting aberrations they achieve an axial PSF shorter by a factor of 1.8, using two cycles of this modal wavefront sensor applied to low order aberration modes. Obviously, the degree of correction required and the profit of this method depend upon the nature of each individual specimen.

Another different way to apply AO in confocal microscopy was done by *Xiaodong Tao et al* [36]. They used a direct wavefront sensor in a fluorescence confocal microscope. Particularly, they implemented a Shack-Hartmann sensor with fluorescent micro-spheres ($1\ \mu\text{m}$ diameter) embedded in the sample as a point source reference beacons. Their set-up was designed to operate in a close-loop. The corrector device was a deformable mirror. A separate laser channel was added to excite the microsphere, which shared the same light path with the imaging channel. The results showed a 4.3x improvement in the Strehl ratio and a 240 % improvement in the signal intensity for fixed mouse tissues at depths of up to $100\ \mu\text{m}$. Although the effects of these microspheres in the live tissue have to be further investigated, this direct method enabled a shorter exposure time during sensing and a higher speed of imaging, which showed its potential ability for live in vivo imaging.

3.2.2 Two-Photon Fluorescence Microscopy

Its intrinsic optical sectioning, larger penetration depth, reduced photo damage as well as other advantages allowed nonlinear microscopy in general, and Two-Photon Fluorescence Microscopy (TPFM) in particular, to become a very important tool in biological imaging since its first presentation by *Denk et al.* in 1990 [16]. As with most AO microscopy techniques, both a direct and indirect wavefront sensing scheme can be deployed for the use with TPFM. Indirect sensing was already explained (section 2.2) and presented (section 3.1 in detail. *Marsh et al.* present the first and fairly simple indirect sensing approach, correcting only for depth induced aberrations as early as 2003 [26]. Again, based on their earlier works ([12, 13]), *Débarre et al.* present another highly sophisticated application of their image based wavefront sensing scheme in 2009 [14]. Since both *Marsh* and *Débarre* essentially use the standard TPFM setup and the image optimization is very similar to the one presented earlier, we will not describe these methods here again. *Rueckel et al.* present a wavefront correction method using coherence-gated wavefront sensing [34] which also beyond the scope of this report. This section will therefor describe direct wavefront sensing based on the work presented by *Aviles-Espinosa et al.* in 2011 [1].

For two-photon fluorescence microscopy, it is not essential to correct for sample or system induced aberrations on the collected beam. Since it is a point scanning technique, all the light emitted in the focus region is collected and only the relative intensity difference is important for the generation of the image (see [28] for a detailed review on TPFM). To achieve the highest possible resolution, especially when imaging deep in a tissue, it is however very important to correct aberrations of the excitation beam. This will not only result in a better, i.e. smaller focus spot, but will also highly increase the efficiency of the nonlinear process. As described in earlier sections, a indirect wavefront sensing is usually applied for microscopic applications. While indirect sensing has several advantages over direct sensing (see sec. 2.2), they have one important drawback that can ultimately not be overcome. They all depend on an iterative optimization procedure to estimate and correct for the aberrations. While random optimization requires many iterations (in some cases up to 3000 iterations per mirror actuator [40]) other methods base the iterations on complicated models and are able to achieve indirect sensing with as little as $2N + 1$ iterations (where N is the number of corrected aberration modes) [12, 13, 14]. However, even these methods require the sample to be exposed and imaged multiple times. To correct 11 aberrations modes (as done in [14]), one needs 23 exposure to acquire an aberration corrected image. While this is suitable for some systems such as CARS where photobleaching is not an issue, it often limits the feasibility of AO in fluorescence imaging. Being aware of these limitations, *Aviles-Espinosa et al.* therefore developed a direct sensing scheme.

As described in section 2.1, to be able to use a wavefront sensor, one needs a point like reference source which is then used to detect the aberration. This point like source, called “nonlinear guide star” (NL-GS) by the authors¹, can be artificially created and imbedded in the sample, as done for standard fluorescence microscopy [2]. This however can cause damage to the sample or might influence the behavior of living samples, limiting its potential for in vivo imaging. *Aviles-Espinosa et al.* realized

¹The term “guide star” is used in reference to astronomy. There a star or an laser spot projected in the sky is used as the reference and called guide star.

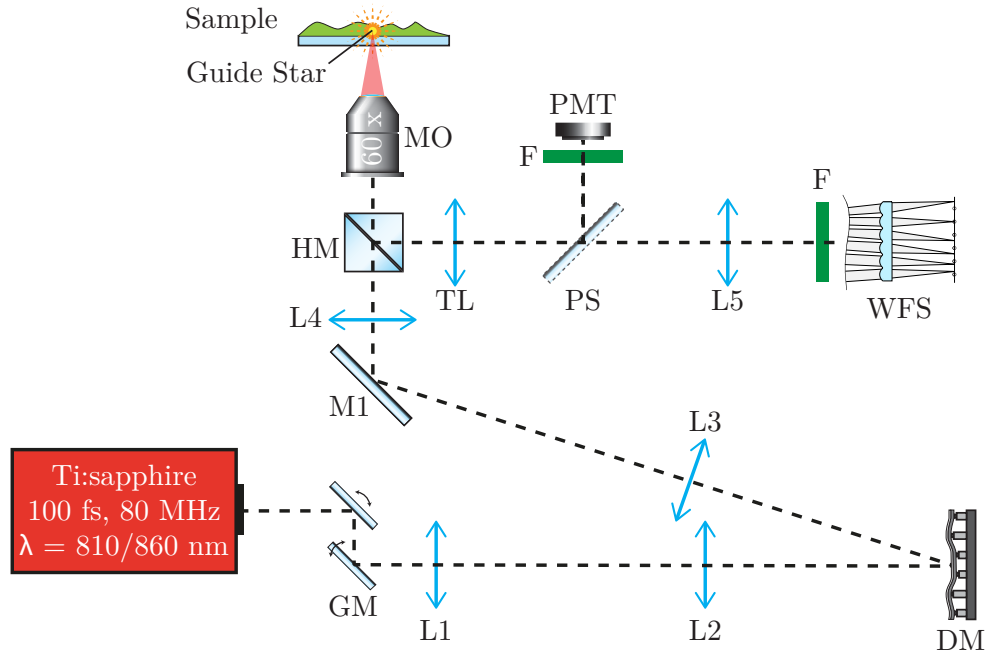


Figure 10: Experimental setup for aberration corrected two-photon fluorescence microscopy as proposed by *Aviles-Espinosa et al.*. GM - galvanometric mirrors, L1-L5 - lenses, DM - deformable mirror, M1 - mirror, HM - filter and beamsplitter, MO - microscope objective, TL - microscope tube lens, F - band pass filters, PMT - photo multiplier tube, WFS - Shack-Hartmann wavefront sensor. The microscope output port is manually selected either for the PMT or for the WF sensor using PS. See [1] for the original image as well as a detailed description of the working principle.

that two-photon excited fluorescence naturally produces a small confined volume which can be used as the guide-star. The setup presented by the authors is shown in Fig. 10. The setup shown is basically an inverted microscope, modified to be used as laser scanning TPFM (see paper for detailed component description and working principle). A mode locked Ti:sapphire laser ($\lambda = 810\text{ nm}$ & 860 nm , pulse duration = 100 fs , repetition rate = 80 MHz , average powers in sample plane = 1.5 mW to 5.6 mW) is used as the excitation beam. The wavefront sensing is performed using a Shack-Hartmann WaveFront Sensor (SH WFS), located at one of the output ports of the microscope. The aberration correction is realized with an electromagnetic Deformable Mirror (DM).

Before the authors started their experiments on biological samples, they first showed that the guide star is reproducible, reliable and independent from the excitation beam aberrations. They continued to verify that the NL-GS behaves as point source as well as proving that aberrations are similar in the complete Field Of View (FOV). Since all these requirements were met, it was show that aberrations in the imaged area can be effectively corrected using only one NL-GS. The authors then continued to calibrate their system, eliminating the passive aberrations of the microscope system coming from the optics for excitation beam as well as the beam path from the objective pupil to the output ports of the microscope. These so called coupling aberrations only need to be corrected once for a given microscopic setup. They were measured and taken into account as a reference for all the subsequent wavefront measurements. The second correction step was to measure the so called focusing aberrations caused by the focusing part of the system. This step was performed every time prior to the actual image acquisition to account for the specific measurement (i.e. the objective, the refractive index matching oil, the cover slip and the sample).

The authors then investigated the performance of their AOM system using both *Caenorhabditis elegans* and mouse brain samples. -For small aberrations and weak scattering only a modest improvement in signal intensity was shown. At an imaging depth of $25\text{ }\mu\text{m}$, the measured signal enhancement

was 1.75x by correcting the coupling aberrations, and 3.61x when focusing aberrations were corrected as well. Similar values were obtained imaging deeper into the tissue. *Caenorhabditis elegans* tissue scatters only weakly and hence spherical aberration is the main aberration for imaging deep into the tissue. Since these should be easily corrected using the presented scheme, the authors were surprised by these relatively small improvements in the signal quality. By using an additional agar pad to simulate moderate scattering as well as imaging deeper into the tissue, the authors investigated the correction efficiency further. Deep in the sample ($127\text{ }\mu\text{m}$) where large sample induced aberrations are prominent, improvements of the signal intensity by a factor of 22.59 were possible, as shown in Fig. 11. It is noteworthy, that the aberration correction increases the signals local maxima while the local minima remain unaltered.

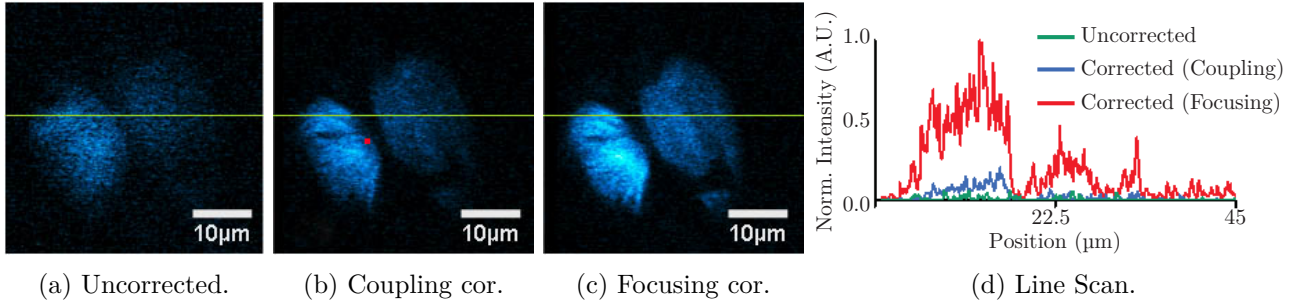


Figure 11: In vivo *C. elegans* sample imaged at $127\text{ }\mu\text{m}$ depth. (a) shows the uncorrected image of a worm section, (b) displays the same section with coupling correction applied and (c) shows the section when both coupling and focusing aberrations are corrected. (d) shows the intensity profile along the green line in the images for all three correction cases. The correction of coupling aberrations improves the signal intensity by a factor of only 1.94 whereas the correction of both coupling and focusing aberrations results in a very good improvement of 22.59. Image after [1].

Finally, the authors investigate the AO system performance using strongly scattering mouse brain tissue. They show that a correction is still possible, but it is less efficient than for moderately scattering samples. It is furthermore possible to record aberration corrected images with a single exposure, no need for complex optimization algorithms and without further sample preparation (i.e. no fluorescent beads need to be inserted). This minimize photobleaching effects, photo-toxicity and limits negative effects to the living sample. Since no model is needed to correct the aberrations, the method is robust and can be applied to fixed and in vivo biological samples. An overall intensity improvement of more than one order of magnitude was shown in some cases. In conclusion, the authors present a flexible and versatile application of adaptive optics in microscopy which can be used in wide range biological imaging applications where a high resolution is required.

4 Conclusion & Future Prospects

Adaptive Optics have been applied in many fields for more than fifteen years, however, in biological imaging it is relatively new to use AO systems. It still needs more research before AO can become a regular component of laboratory microscopes, the particular characteristics of microscopes make AO specially difficult to implement it.

The first think it is important to remark in AO applied to microscopy is that correcting aberrations does not give us high resolution or superresolution by itself. It gives us an improvement in axial and lateral resolution as well as an increase in signal intensity, in other words, AO extend the capabilities of high-resolution or superresolution techniques. Moreover, the advantages of correcting aberrations will always depend on the specimen under study and the microscopy technique used. One of the most critical issues in AO applied to microscopy is the accuracy in the sensing process, that is, how we obtain the aberration information from the sample. It seems to be that indirect sensing is the first option in almost all the experiments for AO microscopes, that is because it is more suitable and easy to

implement in microscopy since only requires a deformable mirror, although it is less faster than direct sensing. Regarding the choice of a direct or indirect methods, we can say in general terms that, if we care more about the speed of measurement and photobleaching is a problem, direct sensing is likely to be the right choice. But, if care more about the signal intensity and photobleaching is not a problem in our experiment, indirect sensing might be the right choice. Although at the end, the right choice will be determined by the microscopy technique as well. Despite all this, further improvements in the sensing process will extend the microscope's ability to image samples with large and more complex aberrations.

Another important aspect in AO for microscopy is that aberrations don't change quickly when we just consider a small region of a biological sample. In this sense, the time response of the current corrector devices is enough. However, as long as we want to scan different parts of the sample, aberrations can change significantly because the refractive index of the specimen varies throughout its volume. Thus, we need to implement in a microscope a faster corrector device or multiple corrector devices in the same AO system. This last solution still needs to be further investigated in microscopy. Also, to our knowledge, AO applications in techniques such as STORM/PALM need still to be experimented. Finally, another future prospect that is essential for live imaging is that further improvements in both, the speed of the sensing process and the time response of the corrector devices, will extend the ability to correct aberrations in real time and with less exposure of the specimens during the measurement.

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