2. Adaptive Optical Microscopy using Direct Wavefront Sensing

2.1 Introduction

In this chapter we will review adaptive optics (AO) in biological imaging using direct wavefront measurement. Here light from a point source in the specimen is used to measure the wavefront with a detector such as a Shack-Hartmann wavefront sensor, similar to the approach that is used in astronomy. The benefit of direct wavefront measurement relative to the sensorless methods discussed in Chapter 2.1 is that the wavefront can be measured quickly in one step. Typically sensorless methods are iterative, requiring a number of measurements. Taking multiple measurements can take more time and may expose the sample to more light which can lead to photo-bleaching. Another benefit is that some indirect methods use optimization of a merit function such as image sharpness or image intensity. In direct wavefront sensing the wavefront aberration is directly measured and corrected rather than optimized. As we shall discuss in this chapter, a common metric for direct wavefront measurement and correction is the Strehl ratio which is defined as the ratio of the on-axis beam intensity to the diffraction limited beam intensity. The Strehl ratio indicates how close the imaging is to the diffraction limit. The objective is getting as close as possible to the diffraction limit. The price that is paid for direct wavefront sensing is system complexity and cost for the addition of the wavefront sensor. In addition it is necessary to provide a point source reference for measuring the wavefront. In this chapter we will discuss how this has been done in different forms of microscopy (wide-field, confocal and multiphoton) after a brief introduction to the general field of wavefront measurement in astronomy and biological imaging.

2.2 Background

2.2.1 Wavefront Sensing & Correction in Astronomy

AO has been used for applications in astronomy\(^1\),\(^2\),\(^3\) vision science\(^4\),\(^5\) and microscopy\(^6\),\(^7\),\(^8\). In astronomy, AO has been used to overcome the image aberrations caused by the Earth’s atmosphere. Light from a distant star, which can be considered a point source since it is so far away, travels through the vacuum of space as a spherical wave. When the wavefront enters Earth’s atmosphere, which is initially nearly planar for the small section that is collected by the telescope, the wavefront is distorted due to dynamic changes in the index of refraction of the atmosphere caused by winds and temperature fluctuations. These fluctuations in the index of refraction cause changes in the velocity of the wavefront, so that some portions travel faster than others, leading to the distorted wavefront shown in Figure 2.1. These dynamic distortions are what cause stars to appear to twinkle. When the star is imaged in a telescope, it appears as a fuzzy blob rather than a point of light, as shown in Figure 2.1(a). By measuring the wavefront distortions from the star using a wavefront sensor\(^9\),\(^10\) the opposite shape of the wavefront distortion can be applied to an adaptive mirror to correct the reflected image, as shown in Figures 2.1(b) and 2.1(c). When a star is used as a reference point source for making wavefront corrections, it is called a “guide star.” If light from a nearby galaxy travels through the same part of the atmosphere, the guide-star can be used to correct the image of the galaxy, as shown in Figure 2.1(b).
Figure 2.1. AO in astronomy. (a) The image of a star appears as a fuzzy blob in a telescope due to the wavefront aberrations caused by turbulence in the atmosphere. (b) If a deformable mirror is used to correct the image of the star to make it back into a point of light, a nearby galaxy with more complex structure will also be corrected. (c) The wavefront distortions can be measured with a wavefront sensor. The wavefront distortions measured using the guide star are fed back through a control system to deform the adaptive mirror to a shape that is the opposite of the distorted wavefront, correcting the reflected wavefront of the “guide star” back into a plane wave. (Credit: Claire Max, Center for Adaptive Optics).

Figure 2.2. The use of AO to correct image aberrations. (a) Astronomy: Neptune observed in the near-infrared (1.65 microns) with and without AO. (Credit: C. E. Max et al., reproduced by permission of the America Astronomical Society). (b) Vision science: Imaging of individual rods and cones in the living human retina. (Credit: Y. Zhang, S. Poonja and A. Roorda, reproduced by permission of the Optical Society of America).

Examples of the use of AO in astronomy and vision science are shown in Figure 2.2. An image of the planet Neptune is shown in Figure 2.2.2(a) with conventional optics and corrected with AO. Images of clouds on the planet can be seen in the corrected image. An uncorrected and corrected image of a living human retina is shown in Figure 2.2(b). The mosaic of the individual rods and cones can be seen in the corrected image. The goal for the use of AO in both astronomy and vision science is to obtain diffraction limited imaging given by the Rayleigh criterion:

\[
\sin(\theta) = 1.22 \frac{\lambda}{D} \quad [2.1]
\]

Where \(\theta\) is the angular resolution, \(\lambda\) is the wavelength of light that is being imaged and \(D\) is the diameter of the aperture that is used for imaging. A commonly used metric used for characterizing how close the image is to the diffraction limit is the “Strehl ratio”, which
is defined as the ratio of the measured on-axis beam intensity to the diffraction limited intensity. An equation for the Strehl ratio $S$ is:\textsuperscript{15}

$$S = e^{-\left(\frac{2\pi \sigma}{\lambda}\right)^2} \quad [2.2]$$

Where $\sigma$ is the root-mean-square deviation of the wavefront and $\lambda$ is the wavelength. An unaberrated optical system would have a Strehl ratio of 1, but typically an optical system with a Strehl ratio greater than 0.8 is considered diffraction limited.

Very often there are no guide stars located sufficiently close to the galaxy of interest in astronomical imaging such that light from the guide star travels through the same part of the atmosphere as the light from the galaxy, so an “artificial guide star” is created where it is desired by exciting fluorescence in the layer of sodium atoms in the Earth’s mesosphere at an altitude of approximately 100 km. A powerful laser (sodium D2a line at 598 nm) is projected upward from the dome of the telescope (after checking to make sure there are no airplanes in the vicinity that would be blinded by the light), as shown in Figure 2.3.

![Figure 2.3. Artificial guide stars in astronomy and biology. (a) A sodium laser is projected up from the dome of the telescope. Credit: Keck II (photography by Laurie Hatch).\textsuperscript{16} (b) Where the sodium laser intersects a layer of sodium atoms in the Earth’s mesosphere, at an altitude of approximately 100 km above the Earth’s surface, the laser excites fluorescence in the sodium layer creating a small point of light that can be used as a guide star. (Credit: La Palma Observatory). (c) In biology, fluorescently labeled features such as centrosomes can be used as guide stars for wavefront measurements in microscopy. (Credit: Prof. Roy, McGill University).\textsuperscript{17}]

### 2.2.2 Wavefront Sensing & Correction in Biology

In biological imaging, there very often are no point-like “natural guide stars” that can be used for making wavefront measurements,\textsuperscript{6} so they need to be created. An example of a guide star that has been used previously for making wavefront measurements to characterize the “point spread function” (PSF) of a microscope is a fluorescent bead.\textsuperscript{18,19} This technique does not require the use of a sub-resolution object to obtain the three-dimensional microscope PSF, so that a bright fluorescent bead that is larger than the diffraction limit of the microscope can be used. Because the wavefront measurement using a Shack-Hartmann wavefront sensor (SHWS) is determined by the motion of spot centroids, the object size is not limited by the diffraction limit of the objective. An example of a SHWS is shown in Figure 2.4 (left).
Figure 2.4. (Left) Shack-Hartmann wavefront sensor. An aberrated wavefront (red) is incident on an array of lenslets (blue), forming an array of images of the guide star on a charge-coupled device (CCD) camera placed at the focal length \( f \) behind the lenslet array. If there is a local slope to the wavefront in a sub-aperture, as indicated by the arrows, the spot will be shifted by an angle \( \beta \). By measuring the shifts in the centroids of the spots, the local slope of the wavefront can be determined. (Credit: Laser Lab Göttingen/Germany). (Right) Hartmann spots from a fluorescent bead.

The aberrated wavefront can be regenerated from the displacement of the “Hartmann spots.”20 Some typical Hartmann spots from a fluorescent bead are shown in Figure 2.4 (right). The spot displacement in each sub-aperture is directly proportional to the product of the mean slope and the focal length of the lenslet. The image is processed to obtain the location of the Hartmann spots by using a cross-correlation centroiding algorithm.21 The slope measurements are then processed to obtain the wavefront using a Fast Fourier Transform (FFT) reconstruction algorithm.22 If the opposite shape of the aberrated wavefront is placed on an adaptive mirror, such as shown in Figure 2.1(c), the reflected image is the corrected wavefront. The wavefront sensor and the adaptive mirror can be used in a closed-loop system as shown in Figure 2.1(c), where the wavefront is measured and an error signal is sent to modify the shape of the mirror to flatten the wavefront (i.e. to bring the Hartmann spots into the center of each sub-aperture on the CCD camera), or in open-loop by characterizing the mirror so that a precise shape that is the opposite of the measured wavefront can be directly placed on the mirror. The open-loop approach provides a faster correction than closed-loop operation where an iterative approach to wavefront measurement and correction is used, but it requires a well calibrated mirror.23,24

There are various ways of estimating a wavefront from the Hartmann slopes.25,26 Two essential pieces of information are needed: (1) the phase difference (the slope measurement times the sub-aperture size) from each sub-aperture and, (2) the geometrical layout of the sub-apertures. The wavefront can then be calculated by relating the slope measurement to the phases at the edge of the sub-aperture in the correct geometrical order. A method for directly obtaining the deformable mirror commands from wavefront sensor measurements is described by Tyson.27 First a mask with the sub-apertures must be created; this will generate the geometric layout of the sub-apertures in the aperture. The next step is to measure and record the response of all the sub-aperture slope changes while actuating each actuator. The results will be a set of linear equations which shows the response of the wavefront sensor for each of the actuator commands that is called the “poke” matrix (also called the actuator influence matrix). The deformable mirror (DM) commands can then be obtained by solving the following equation:
\[ s = Av \]  \[2.3\]

Where \( s \) is an \( n \) size vector obtained from the SHWS slope measurements, \( v \) is an \( m \) size vector with the DM actuator commands, and \( A \) is an \( n \times m \) size poke matrix. In the linear approximation, Equation 2.3 can be pseudo inverted to obtain an estimate of the DM command matrix. Note that some DMs are nonlinear devices so that applying a large change in voltage to an actuator will not necessarily result in the same change in shape every time, but the matrix given in Equation 2.3 performs well in a closed-loop system since only very small voltage changes occur in each feedback cycle thus reducing the nonlinear effects. There are various methods for inverting the matrix \( A \) including singular value decomposition (SVD). The advantage of using SVD is that the mode space can be directly calculated. The noisier modes, and all the null space modes by default, can then be removed by setting a threshold on the singular value space.\(^{26,28}\)

To use this approach for making wavefront measurements in wide-field microscopy, we have injected fluorescent bead “guide stars” into the samples that are then used for making SHWS measurements.\(^{29,30,31,32,33}\) An example of fluorescent beads (microspheres) that have been injected into a fruit fly embryo is shown in Figure 2.5 (left). Each of these beads can be used as an artificial guide star for making a wavefront measurement. It is also possible to use a fluorescent bead that is excited at a different wavelength than the labeling that is used for imaging the sample to minimize photo-bleaching of the sample, so long as the correction at the guide star wavelength works for the correction of the emission light from the sample labeling.\(^{33,34}\) This will depend on the dispersion of the tissue. So far tissue dispersion has not been an issue.

![Figure 2.5](image1.png)

**Figure 2.5.** (Left) Fluorescent microspheres injected into a fruit fly embryo for use as fluorescent guide stars in wide-field microscopy. Combination of a differential interference contrast image (DIC) and a confocal image of injected microspheres in fruit fly embryo 40 \( \mu \)m below the surface of the embryo.\(^{29}\) (Right) Aberrations that were measured for a 1 \( \mu \)m fluorescent bead at a depth of 45 \( \mu \)m below the surface of a Drosophila embryo.\(^{31}\)

Figure 2.6 shows the design of the system used to measure the wavefront aberration introduced by a Drosophila embryo. Two different objectives 20x and 40x (Melles Griot, Rochester, NY) were used with numerical apertures of 0.4 and 0.65 respectively. L1 and L2 are 65 mm focal length lenses that image the aperture of the objective (plane P1) onto the Shack-Hartmann wavefront sensor (plane P2). The field-stop between L1 and L2 blocks the light coming from other microspheres in the field of view, allowing only the
light from one microsphere to pass. The field-stop could be moved further down the optical system since it is only needed by the Shack-Hartmann sensor. The minimum size of the field stop was set to 1 mm in image space to prevent excessive spatial filtering of the wavefront.\(^{35}\) The large distance between L1 and the aperture (P1) allows for the excitation laser (HeNe \(\lambda = 633\) nm) to be placed in this area. The laser is directed to the optical path via the 45° beam splitter BS1 (Semrock, Rochester, NY). An emission filter was also added after L2 to reduce the effect of scattered laser excitation light by the embryo and to allow for the Shack-Hartmann wavefront sensor to only see the fluorescent emission light. By using a 90/10 beam splitter the microsphere can be simultaneously imaged by the Shack-Hartmann sensor and the science camera (S Camera). Immediately following the beam splitter is the wavefront sensor which is composed of a lenslet array (AOA Inc., Cambridge, MA) and a cooled CCD camera (Roper Scientific, Acton, NJ). The lenslet array has 1936 (44x44) lenses, each with a focal length of 24 mm and a diameter \(d_{L3}\) of 328 \(\mu\)m. The lens L3 de-magnifies the pupil by a factor 2 so that it can fit into the cooled camera.

![Figure 2.6](image_url) Microscope set up with a Shack-Hartmann wavefront sensor. Beam splitter BS1 allows the laser light to be focused onto the sample. Beam splitter BS2 allows for both the science camera and the WFS to simultaneously see the fluorescent microsphere.\(^{29}\)

The aberrations that have been measured for a fluorescent bead at a depth of 45 \(\mu\)m below the surface of a Drosophila embryo are shown in Figure 2.5 (right). The guide star reference source used to measure the wavefront was one of the fluorescent microspheres shown in Figure 2.5 (left). The aberrations are expressed in terms of Zernike polynomials, a sequence of orthogonal polynomials defined on a unit disk.\(^{36}\) The Zernike mode number corresponds to different polynomials (0-piston, 1-tip, 2-tilt, 3-defocus, 4-astigmatism \(0^\circ\), 5-astigmatism \(45^\circ\), 6-X coma, 7-Y coma, 8-3\(^{rd}\) order spherical) with names that are indicative of the type of aberration. In general, as the order increases, the spatial frequency of the polynomial increases. As can be seen in Figure 2.5 (right), the aberrations tend to decrease with increasing order.\(^{37}\) Here astigmatism and other spherical aberrations dominate the wavefront error. This is mainly due to the index mismatches in the optical path as well as the curved body of the embryo, which mostly introduced lower-order aberrations. The optical aberrations due to the cover slip and air-glass interface, including tip, tilt and focus, have been removed using a reference image with a guide star but without a biological specimen. The Zernike mode decomposition for a variety of other biological specimens has been measured and follows the same trend of decreasing aberration at higher order.\(^{37}\)
One of the challenges in designing a SHWS is the amount of light the reference source can provide. Fluorescent microspheres are composed of fluorescent dye. Since the amount of light emitted is proportional to the radius cubed, smaller beads provide less light. The size of the beads should be smaller than the diffraction limit of one sub-aperture of the Shack-Hartmann wavefront sensor. Note that this is larger than the diffraction limit of the microscope aperture by the ratio $D/\text{d}_{\text{LA}}$ (lenslet array diameter). Since the diffraction limit of the microscope is inversely proportional to the numerical aperture (NA) smaller beads are needed for higher numerical aperture systems. Fortunately the light gathered by the objective also increases with increasing NA (light gathering power $\sim$NA$^2$). Thus, increasing the wavefront sampling by a factor of 4 increases the size of the microsphere radius by a factor of 2, and the amount of light emitted by a factor of 8. The only way to determine if a microsphere, or any fluorescent source, will work is to image it with a SHWS using an objective, as shown in Figure 2.6. In order to increase the speed of the AO correction loop the bead size should be maximized.

The size of the bead $d_{\text{bead}}$ should be smaller than the diffraction limit of the wavefront sensor when imaged through the microscope objective:

$$d_{\text{bead}} < d_{\text{diffraction limit}} = 2.44 \frac{\lambda}{2\text{NA}_{\text{ob}} \text{d}_{\text{LA}}} = d_{\text{DLO}} \times \frac{N_{\text{P}}}{d}$$

Where $\lambda$ is the wavelength at which the fluorescent beads are emitting, $\text{NA}_{\text{ob}}$ is the Numerical Aperture of the objective, $D_o$ is the limiting aperture of the objective, and $\text{d}_{\text{LA}}$ is the diameter of the lenslets in the array. This could also be represented as the diffraction limit of the objective $d_{\text{DLO}}$ times the number of sub-apertures across the limiting pupil. Using this technique we can measure the aberration introduced by a biological sample by injecting a fluorescent bead into the sample.

2.3 Techniques

2.3.1 AO Wide-Field Microscopy

To make both wavefront measurements and corrections, an AO system was added to the back port of an Olympus IX71 inverted microscope (Olympus Microscope, Center Valley, PA) as shown in Figure 2.7. The AO system was designed around an Olympus 60x oil immersion objective with a numerical aperture of 1.42 and a working distance of 0.15 mm. Lenses L1 and L2 have 180 mm and 85 mm focal lengths, respectively, and are used to image the back pupil of the 60x objective onto the deformable mirror (DM) (Boston Micromachines, Boston, MA). The DM has 140 actuators on a square array with a pitch of 400 $\mu$m, a stroke of 3.5 $\mu$m and a 4.4 mm aperture. L3 and L4 are 275 mm and 225 mm focal length lenses, respectively, and are used to reimagine the back pupil of the objective onto the Shack-Hartmann Wavefront Sensor (SHWS). The system has an illumination arm that couples laser light in through a dichroic mirror D. A confocal illuminator (not shown) is used for excitation of the guide star reference beads. This confocal illuminator allows the illumination of a single guide star fluorescent bead to create a single spot. The beam splitter (BS) lets 90 percent of the emitted light coming from the guide star go to the SHWS for wavefront measurement and 10 percent for imaging in the Science Camera (SC). The SHWS is composed of a 44x44 element lenslet array (AOA Inc., Cambridge, MA) and a cooled CCD camera (Roper Scientific, Acton, NJ).
Figure 2.7. AO wide-field microscope. (Left) Schematic diagram of the optical system. The back pupil (BP) plane of an objective lens is imaged onto a deformable mirror (DM) by the relay telescope formed by lenses L1 & L2. After reflection from the DM, the light is reflected off of a fold mirror (M) and is imaged onto a Shack-Hartmann wavefront sensor (SHWS) through the relay telescope formed by lenses L3 & L4. The SHWS is comprised of a lenslet array (LA) and a CCD camera. A beam splitter BS directs some of the light onto a second CCD camera that captures the image (science camera SC). A laser illuminates the sample to cause the guide star to fluoresce. Laser excitation light is coupled in through a dichroic mirror D. (Right) Implementation of the AO wide-field microscope.32,33

2.3.1.1 Results

Demonstration of wavefront correction is shown in Figure 2.8, where each panel shows the results of an additional correction step taken 10 milliseconds apart. Each correction was done using the light coming from a single bead to directly measure the wavefront. The measurement was then fed back to the deformable mirror by using a proportional gain of 0.4 which was the highest possible gain for this sample before the onset of oscillations (Lyapunov stability criteria).38 In AO DM correction usually requires a gradual change in shape to account for the nonlinearity of the wavefront sensor and DM. This results mainly from the nonlinear effects of the DM and secondly, usually much smaller, the nonlinear effects of the SHWS. The nonlinear effects of the DM come from the nonlinear dependence of the electrostatic actuation force on the applied voltage and plate separation for a parallel plate actuator and the nonlinear restoring force from stretching of both the mechanical spring layer as well as the mirror surface.39 Figure 2.8(a) shows the original point spread function (PSF) of the microsphere taken with the science camera before correction. Figure 2.8(b) shows the result of correcting for 40% of the measured wavefront error in Figure 2.8(a). These steps were repeated until there was no additional significant reduction in wavefront error (i.e. less than 7 nm). Figure 2.8(e) demonstrates the results of correcting the wavefront after 4 steps in the AO loop. Each image has been normalized to its own maximum to clearly show the details of the PSF. The bar in Figure 2.8(e) is approximately equal to the diffraction limit of the 40x objective, 0.45 μm. The improvement in the Strehl ratio was approximately 10x. As can be seen, the original PSF in Figure 2.8 does not look like a point, but does after wavefront correction in Figure 2.8(e). This improvement is important for imaging features at the diffraction limit of the microscope, and for use in deconvolution software that assumes an ideal PSF.
The benefit of adaptive optical correction can be seen when small fluorescent beads (1 μm green fluorescent beads (Invitrogen, Carlsbad, CA)) are spaced closely so that the individual beads cannot be resolved, as shown in Figure 2.9 (left). After AO correction, the individual beads can be resolved in Figure 2.9 (right). This figure is similar to images of closely spaced stars in astronomy. Without AO the stars are not resolved, but can be resolved with the use of AO.

### 2.3.1.2 Isoplanatic Angle

The isoplanatic angle is a relative measure of the field of view over which the AO system can correct the wavefront aberration to an acceptable level\(^1\) (~1 rad\(^2\) residual wavefront error):

\[
\sigma_{\theta}^2 = \left\langle \left( \varphi(X,0) - \varphi(X,\theta_0) \right)^2 \right\rangle = 1 rad^2 \quad [2.6]
\]

Where \(\varphi\) is the wavefront in radians, \(X\) is a vector representing the two-dimensional coordinates, \(\theta_0\) is the isoplanatic angle, and \(\sigma_{\theta}^2\) is the mean-square error between the measured and observed wavefront. We can determine the isoplanatic half-width by multiplying the isoplanatic angle by the focal length of the objective. In order to determine the isoplanatic angle we took wavefront measurements from two microspheres separated by a distance \(d\). A microsphere was excited by shining a laser on it. Each microsphere was excited individually. Each wavefront sensor measurement was collected over a period of 500 milliseconds, much longer than the typical AO loop bandwidth (~50 ms). This insures that there is little noise in the data. The standard deviation for each individual wavefront was measured to be better than 1% of the wavelength at 647 nm. Table 2.1 shows three different measurements taken with a 40x (0.75 NA) objective lens.
Table 2.1. Isoplanatic angle measurements for the 40x magnification, 0.75 NA objective lens.31

<table>
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<tr>
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<th>1</th>
<th>0.7</th>
<th>1.60</th>
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<td>1.24</td>
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<tr>
<td>3</td>
<td>25</td>
<td>19.1</td>
<td>1.69</td>
<td>1.10</td>
<td>1.30</td>
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Mean 19±5.57 14.5±4.25 1.42±0.39 1.41±0.43 0.93±0.32

The first measurement shows that the wavefront error for the bead located at the center of the field of view RMS(1) is 1.60 radians, the wavefront error for the bead located 14 μm from the center RMS(2) is 1.90 radians, and the wavefront error between the two measurements RMS(1-2) is 0.73 radians. Taking the average of three measurements shows the isoplanatic half width is 19±5.6 μm. This results show that a reference microsphere together with an AO system can help to improve the quality of the images taken, not just at the location of the microsphere but also within a circle 10 microns in radius from the location of the microsphere.

An emerging field is tomography AO, where multiple light sources together with multiple SHWS’s are used. The information from each wavefront sensor is then processed using a reconstructor to acquire a tomographic image of the changes in the index of refraction in the optical path.1 One of the advantages of using tomography AO is that it can provide information on the depth dependence of variations in the index of refraction in the tissue which allows for the AO system to correct for the wavefront aberrations only in the optical path. This technique can also extend the isoplanatic angle by correcting wavefront aberrations that are common to a larger field of view. By depositing multiple fluorescent beads into the biological sample and using multiple wavefront sensors we can also apply the tomographic techniques that have been developed for astronomical AO.41

Multi-conjugate AO uses multiple wavefront correctors that are placed conjugate to multiple layers of wavefront aberration, rather than the more typical approach in biological imaging where adaptive optical correction is accomplished with a single deformable mirror DM in a plane conjugated to the pupil of the microscope objective lens that is used for imaging.1 Using multi-conjugate AO, the isplanatic patch can be widened. This approach has been implemented in astronomy, vision science, and is now being investigated for use in biological imaging. In astronomy there are relatively well defined layers in the atmosphere that can be conjugated to wavefront correctors (e.g. ground layer, high altitude). In vision science one plane was conjugate to the pupil of the eye while another plane was conjugate to the retina. In biological imaging the aberrations are in general spread throughout a volume, although for some samples the aberrations may be concentrated within a particular plane.

In scanning laser microscopy, AO correction over large volumes was accomplished using a laser-induced guide star and direct wavefront sensing. They are able to demonstrate adaptive correction of complex optical aberrations at high numerical aperture (NA) with a 14 ms update rate. This enables recovery of diffraction-limited imaging over large volumes (>240 μm per side).53

2.3.2 AO Confocal Microscopy
A similar approach for measuring wavefront aberrations with a Shack-Hartmann wavefront using guide star reference beacons can also be implemented in confocal microscopy.54,55 A benefit of scanned laser microscopy is that a similar approach to the laser guide stars that are used in astronomy can be implemented in biological imaging without the need for injection of fluorescent beads. Biological samples are typically labeled with fluorescent markers which can be illuminated by the scanned laser beam to generate a small point of fluorescent light for use as a guide star. As described above, in astronomy the atomic sodium layer in the Earth’s mesosphere can be used to generate an artificial guide star, as shown in Figure 2.3. The small point of fluorescent light that is excited where a sodium laser intersects the sodium layer can be used as a reference for making wavefront measurements with a wavefront sensor. Even though the sodium layer is extended around the Earth, only the small spot where the laser penetrates the sodium layer is excited, resulting in a small spot. In biological samples, the intersection of a focused laser beam with a fluorescently labeled structure can result in a similar small spot of light for wavefront measurements. The only requirements are that light from other focal planes are rejected and that the resulting spot of light is smaller than the diffraction limit of the wavefront sensor, which is considerably larger than the diffraction limit of the microscope imaging system as shown in Equation 2.4. Another approach uses backscattered light rather than fluorescence; however this double-pass configuration leads to lower sensitivity to odd-symmetry aberration modes.56

An example of a scanning laser confocal microscope with AO is shown in Figure 2.10. A solid state laser (λ=515 nm) provides the excitation light for both fluorescence imaging and wavefront sensing. The light is fed into an objective lens (60x water objective, NA 1.2, Olympus) and scanned on the sample in a raster pattern with two galvo scanners (6215H, Cambridge Technology). The emission light from yellow fluorescent protein (YFP) is divided by a beam splitter (BS). Half of the light is collected by a photomultiplier tube (PMT, H422-50, Hamamatsu). The other half of the light is used for wavefront sensing. To eliminate the intensity loss from the division of light by the BS, a switchable mirror can be included instead of the BS to maximize the signal into the PMT or the wavefront sensor. The AO system includes a deformable mirror (DM, 140 actuators, Boston Micromachines) and a Shack–Hartmann wavefront sensor (SHWS). The SHWS is composed of a 44×44 element lenslet array with a lenslet diameter of 400 μm and focal length of 24 mm (AOA Inc., Cambridge, Massachusetts) and an electron multiplying (EM) CCD camera (Cascade II, Photometrics). In order to minimize the amount of out-of-focus light that enters the SHWS, irises I1 and I2 are placed in the light path. These irises also block stray light from the DM, scanner, and lenses. The iris acts as a low pass spatial filter. However, the higher-order wavefront will only give a small contribution to the overall aberration.57 For mouse brain tissue, the first 14 Zernike modes (third-order) contribute the most aberration. The wavefront error with an iris can be estimated.18,35 For a 50 μm thick mouse brain tissue sample, the RMS wavefront error with and without an iris is only 0.0549λ (λ=527 nm) when the size of the iris is set to the diameter of the point spread function (PSF) with 80% of the encircled energy (150 μm for I1). This setting will block 53% of the light from a plane within 1.5 μm of the focus. A cross-correlation centroiding algorithm and a fast Fourier transform reconstruction algorithm were implemented to obtain the wavefront.21,22 To make an accurate measurement, the diameter of the guide star should be smaller than the diffraction limit of
the wavefront sensor, defined in Equation 2.4. In our current setup, the diffraction limit for the wavefront sensor is equal to 5.64 μm. Because the fluorescent light from a given point is proportional to the light intensity illuminating that point, the size of the guide star is limited by the illumination PSF, similar to the case in astronomy for a laser guide star illuminating the sodium layer in the mesosphere. The PSF can be calculated from the wavefront measurement. For yellow fluorescent protein (YFP) at a depth of 70 μm, the diameter of the PSF with 80% of the encircled energy is 1.4 μm, which is small enough to be used as a guide star.

**Figure 2.10.** AO confocal microscope. (Left) Schematic diagram of the confocal optical system. The emission light from yellow fluorescent protein (YFP) is divided by a beam splitter (BS). Half of the light is collected by a photomultiplier tube (PMT, H422-50, Hamamatsu). The other half of the light is used for wavefront sensing. The AO system includes a deformable mirror (DM) and a Shack–Hartmann wavefront sensor (SHWS). F1, F2 and F3 are filters for the SHWS, the Solid State Laser and the PMT, respectively. BB is a beam blocker. (Right) Layout of the optical system.

2.3.2.1 Results

The AO confocal microscope has been used to image mouse brain samples with cell bodies and dendrites labeled with YFP to study neural plasticity, and Drosophila to study early development deep within the embryo. For the studies of mouse brain samples, both labeled dendrites and cell bodies have been used as guide stars for wavefront measurements, as shown in Figure 2.11 (right). A fixed brain tissue slice from a YFP-H line transgenic mouse was prepared. YFP is labeled on the cell bodies and protrusions of the neurons. Sample brain coronal sections (100 μm) were mounted with anti-fade reagents (Invitrogen). The spherical aberration induced by the cover plate was compensated by adjustment of a correction collar on the objective lens. The approximate structure of the YFP is initially identified in the confocal image without wavefront correction. The laser beam is then steered to the region of the YFP. The YFP distribution in the background may affect the accuracy of the wavefront measurement. Similar challenges are found for non-uniformities of the sodium layer in Earth’s mesosphere in astronomical laser guide stars. Nonetheless, for the mouse brain tissue studied here, the YFP in the neuron cell body with a diameter of 20 μm provides a good uniform background, where the maximum emission is from the focal plane. For other small structures, a refocusing operation is performed to achieve the best focal plane by maximizing the intensity of the Hartmann spots on the wavefront sensor. The emission light from the focal plane then makes the greatest contribution to the wavefront measurement. The DM corrects the aberration in a closed loop using the direct slope algorithm. The corrected confocal image of the isoplanatic region around the YFP guide star is then captured with the optimal shape on the DM.
Confocal images are collected by scanning along the Z axis with a 3 μm range and a 0.15 μm step size. The final image is achieved by maximum intensity projection applied to the images. After turning on the DM, the wavefront error converges after approximately 10 iterations, which takes ~0.30 s. The YFP on the cell body is used as a guide star and located at a depth of 70 μm, as shown in Figure 2.11(b) (left). The RMS wavefront errors without correction and with correction are 0.35λ and 0.034λ, respectively, as shown in Figures 2.11(c) and 2.11(d) (left). The confocal images before correction and after correction are shown in Figures 2.11(a) and 2.11(b) (right). The intensity profile along the dashed lines across a dendrite and a spine is shown in Figure 2.11(f) (right). The peak intensity increases by 3x. The image of the dendrite and spines is much clearer after correction with improved contrast. The Strehl ratio is improved from 0.29 to 0.96, a significant 3.3x improvement. To validate the proposed method, mouse brain tissues with fluorescent microspheres (1 μm diameter, Bangs Laboratories, Inc.) are prepared. The wavefront error is corrected using the fluorescent protein first. The wavefront is measured again using a nearby fluorescent microsphere, which is located 3 μm from the fluorescent protein. Twenty measurements are made. The average RMS wavefront error on the fluorescent protein and microsphere are 0.0352λ and 0.0348λ, respectively, with variances of $5.9 \times 10^{-7} \lambda^2$ and $1.9 \times 10^{-7} \lambda^2$, respectively. The average RMS difference between these two methods is only 0.0004λ.

The AO confocal microscope has also been used for dynamic live imaging of the Drosophila embryo. Here spatially and temporally dependent optical aberrations induced by the inhomogeneous refractive index of the live Drosophila samples limit the
resolution for live dynamic imaging. We used green fluorescent protein (GFP) guide stars, with GFP-tagged centrosome proteins Polo and Cnn, for live imaging. The results demonstrate the ability to correct the aberrations and achieve near diffraction limited images of medial sections of large Drosophila embryos. GFP-polo labeled centrosomes can be observed clearly after correction but cannot be observed before correction.

The samples, upstream activation sequence (UAS) EGFP-Cnn; Nanos-Gal4 flies and GFP-Polo flies where GFP is cloned into the 5’ coding region of the Polo gene were reared on standard cornmeal and molasses media. Embryos were collected for one hour on grape juice agar then aged at room temperature for 30-60 minutes. Dechorionation was done by hand using double-sided tape and the embryos were adhered to coverslips. Embryos were covered in Halocarbon oil (Sigma) to allow oxygen permeation and inhibit desiccation.

In order to find a suitable guide star, a guide star searching algorithm was developed to localize the desired guide star automatically during the confocal imaging process. The algorithm first initializes the guide star size $R_s$, the threshold value $T$ for image thresholding and the maximum star number $N_{\text{max}}$. The number of detected guide stars depends on these settings. The noise in the image is first removed using Gaussian filters. The location $S_{\text{max}}$ of the global maximum of the image is determined, which is used as the first guide star. The next guide star is located by assigning zero intensity to the area of the previous guide star and searching for the maximum of the modified image. The searching loop stops when the predefined maximum number of guide stars $N_{\text{max}}$ is achieved or the intensity of the star is less than the threshold value $T$. In searching for the best guide star, three criteria are available: the brightest guide star, the star closest to the center of the image and the brightest star in a defined area. To achieve an accurate wavefront measurement, the size of the guide stars should be small enough to provide a diffraction-limited image on the wavefront sensor as defined in Equation 2.5. For our setup, $\lambda = 0.509 \mu m; \ NA = 1.1; \ D_0 = 4\times10^3 \mu m; \ d_l = 400 \mu m$. So $d_{\text{diffraction limit}}$ is equal to 5.64 $\mu m$.

For laser scanning fluorescent microscopy, the emission light from a given point on the FP labeled structure is proportional to the light intensity illuminating that point. If the size of the FP labeled structure is smaller than the illumination PSF, the size of the guide star is equal to the size of the real structure. If the size of the FP labeled structure is larger than the PSF, then the size of the guide star depends on the illumination PSF. In this case, it is like the laser guide star used in astronomy that is excited in the sodium-layer. The only part of the sodium layer that fluoresces is where it is illuminated by the laser. The illumination PSF is defined as:

$$h(x_2, y_2) = \frac{i}{\lambda} \int \int P(x_1, y_1) e^{-ik\varphi(x_1, y_1)} \frac{e^{-ik(r-R)}}{rR} \cos(n, r) dS$$  [2.7]

where $(x_2, y_2)$ are the coordinates in the focusing plane. $(x_1, y_1)$ are the coordinates in the pupil plane. $P(x_1, y_1)$ and $\varphi(x_1, x_2)$ are the light field in amplitude and phase, respectively. $k$ is the wave number. $n$ is the unit normal of the pupil plane. $r$ is the unit vector from $(x_1, y_1)$ to $(x_2, y_2)$. $R$ is the distance from the pupil plane to $(x_2, y_2)$. $dS$ is the area element on the pupil plane. $\lambda$ is the wavelength of the illumination light. The size of the guide star can be determined by the area of the PSF with 80% encircled energy. Owing to the high numerical aperture of the objective lens, the guide star size for the diffraction limit of the wavefront sensor is larger than that of the microscope system in most cases. Due to the short exposure time (~50 ms) for wavefront measurement, the theoretical maximum
speed of the guide star motion in the lateral plane can be as high as 49 μm/s at a depth of 90 μm.

The light from out-of-focus planes adds noise to the wavefront measurement. A spatial filter (SF) can be placed at the focusing plane between the relay lenses in front of the wavefront sensor. However, the SF also removes high-order wavefront from the sample. Fortunately for most biological tissues, higher-order Zernike modes give only a small contribution to the overall aberration.64 For the Drosophila embryo, the first 14 Zernike modes (without piston, tip, and tilt) give the major contributions.33 The size of the pinhole can be determined from the band-limit of the wavefront measurement, which depends on the number of the Zernike modes to be measured.4,35,65 Fourteen lenslets are the minimum number for a reliable measurement of the aberrations up to the first 14 Zernike modes. The measured wavefront is bandwidth limited at \( \frac{d_{\text{sub}}}{2} \) because of aliasing, where \( d_{\text{sub}} \) is the width of the sub-aperture.31 A spatial filter with a width of \( \frac{\lambda}{d_{\text{sub}}} \) can attenuate the high-spatial frequency content above \( \frac{d_{\text{sub}}}{2} \). With an aperture of 4 mm, \( d_{\text{sub}} \) for 14 lenslets is 0.85 mm. The angular size of the spatial filter is \( 6 \times 10^{-4} \) rad for \( \lambda = 509 \) nm, which corresponds to a pinhole size of 150 μm.

The ability for making a wavefront measurement using a fluorescent protein centrosome guide star (FPCGS) was tested for measurement of spatially dependent wavefront aberrations in the Drosophila embryo with EGFP-Cnn labeled centrosomes at four different positions as shown in Figures 2.12(a-d). In this experiment, we are looking at the outer edge of an ovoid-shaped embryo and therefore a small fraction of the distance is through cytoplasm and the other fraction is through the mounting medium. The illumination PSF and corresponding wavefront error show its high dependence on the sample orientation and imaging location. To analyze the specific aberrations, the wavefront measurements can be decomposed into different Zernike polynomials as shown in Figure 2.12(e). In contrast to mouse brain tissue, where spherical aberrations are the dominant aberrations, the curved edge of the Drosophila embryo induces a large amount of astigmatism (Zernike modes 5 and 6) aberrations because of its cylindrical shape. The signs of these modes change according to the location. Those measurements also verified the necessity to correct these spatially dependent aberrations using AO. The results of these corrections are shown in confocal microscopy images without and with corrections that are captured at a depth of 83 μm below the coverslip, as shown in Figs. 2.12(f-g). The GFP-polo labeled centrosomes can be observed clearly after correction but cannot be observed before correction. This illustrates how the use of AO is critical for imaging these features. The size of the PSF decreases from 1.7 μm, before correction, to 0.21 μm, after correction. The Strehl ratio calculated based on the PSF shows an increase from \( 3.3 \times 10^{-3} \) to 0.7.
Figure 2.12. Wavefront measurement and correction. (a-d) The averaged point spread function (PSF) and wavefront errors over 6 measurements using EGFP-Cnn labeled centrosomes of a cycle 14 Drosophila embryo at four different locations (P1, P2, P3 and P4) at a depth of 60 μm. (e) The averaged coefficient value of the first 15 Zernike polynomial modes at these four locations. The error bar is the standard deviation for 6 measurements. (f-g) The images and PSF without and with correction for a cycle 14 Drosophila embryo with GFP-polo at a depth of 83 μm. Scale bars, 2 μm.

The penetration depth of the AO microscope for live imaging of a Drosophila embryo was tested by performing AO correction during Z scanning from the top surface to a depth of 100 μm below the coverslip with a 1 μm z-step size. The AO correction is performed at each z-step. The purpose of the guide star searching algorithm is to search and calculate the location of EGFP-Cnn at each depth, acting as a potential guide star, in a cycle 13 embryo. Figures 2.13(a,b) (Left) show the maximum intensity projection (MIP) produced from a scan series without and with correction, respectively. The GFP at the edge of embryo at different depths can be observed. Before correction, the EGFP-Cnn labeled centrosomes can only be observed down to 60 μm in depth. After correction, they can be observed below a depth of 80 μm. Using the 3D view function in ImageJ with a resampling factor of 2, the 3D images of the Drosophila embryo show the imaging depth increases from 60 μm to 95 μm, with more than a 50% increase in imaging depth as shown in Figs. 2.13(c) and 2.13(d) (Left). Figures 2.13(e) and 2.13(f) (Left) show the enlarged images without and with correction at depths of 60 μm and 90 μm, respectively.
Figure 2.13. (Left) Comparison of the three-dimensional imaging without (left) and with (right) correction for imaging of cycle 13 fly embryos with EGFP-Cnn label. The maximum intensity projection of the scan series from the top surface to 100 μm without (a) and with (b) AO. The 3D reconstructions without (c) and with (d) AO. The confocal images without (e) and with (f) AO at the depths of 60 μm and 90 μm. The color maps are scaled to show the image data over its full range. Scale bar, 10 μm.59 (Right) Two-color confocal imaging with AO provided by a de-scanned two-photon guide star deep in the living zebrafish brain. 3D volume rendering (left) of oligodendrocytes (magenta) and neuronal nuclei (green) from the optic tectum through the midbrain. MIP before (center) and after (right) AO correction across four sub-volumes spanning depths indicated by yellow boxes (left) demonstrate the recovery of diffraction-limited resolution throughout the 200-μm-deep imaging volume.53

Figure 2.13 (Right) is a deeper (200 μm) AO confocal image of the zebrafish brain. The zebrafish is more transparent than the fly embryo making it possible to image deeper into the specimen before scattering becomes the limiting factor for the imaging depth. This work used a two-photon guide star and direct wavefront sensing to demonstrate adaptive correction of complex optical aberrations up to the 45th Zernike mode.53

For the fly embryo the wavefront error is measured at each depth before correction. After correction, the wavefront is measured again, directly from the sample with an updated correction by the DM. PSFs are then calculated from the wavefront measurements. Figures 2.14(a) and 2.14(b) show the wavefront measurement and estimated PSF at a depth of 90 μm below the coverslip without and with correction, respectively. The RMS wavefront errors at different depths with and without correction are shown in Figure 2.14(c). Without correction, the RMS wavefront error reaches approximately 0.8λ, when the imaging depth is 90 μm. The Zernike coefficient values without AO with the change of depth are shown in Figure 2.14(d). Below a depth of 50 μm, the astigmatism and coma aberrations (Zernike index 5, 6, 7 and 8) begin to decrease with the increase of trefoil aberration (index 9). Those contribute a RMS wavefront error of around 0.8 λ from 50 μm to 90 μm. However the increases in the high order aberration (the third order) generates a larger PSF with an increase in depth. The decrease of the Strehl ratio in Figure 2.14(e) shows the degradation of the optical performance with the imaging depth.
After correction, even at a depth of 90 μm, the system can still achieve a Strehl ratio of 0.6 with an RMS wavefront error of 0.1λ. Aside from improving the penetration depth, AO also improves the optical resolution. Although the EGFP-Cnn labeled centrosomes can be observed at a depth of 60 μm without AO, the resolution is still poor because of the aberrations as shown in Figures 2.14(e) and 2.14(f). Before correction, the size of the PSF is 1.67 μm at a depth of 60 μm. After correction, it decreases to 0.22 μm as shown in Figure 2.14(f). At a depth of 90 μm, it shows a significant improvement of the PSF by a factor of nine.

Figure 2.14. Comparison of the wavefront measurements and the PSFs without and with AO for different depths. The wavefront measurements and PSF without (a) and with (b) AO at the depth of 90 μm. The RMS wavefront errors change with the depth (c). The red and blue lines indicate the measurement without and with AO respectively. The Zernike coefficient values without AO with the change of depth (d). The Strehl ratio (e) and PSF (f) size change for different depths. The red and blue lines indicate without and with AO, respectively. (λ = 509 nm).59
Enabled by fast wavefront measurement and correction ability, AO microscopy using fluorescent protein guide stars can be used for time lapse 4D imaging. We recorded EGFP-Cnn labeled centrosomes of an early Drosophila embryo for 4D imaging at a depth of 80 μm with an image size of 512x512 pixels and a time resolution of 30 s for five consecutive focal planes (1 plane/μm) over 20 minutes. At the beginning of each time period, the wavefront error is corrected at the third focal plane. In every time interval, images with and without correction are collected sequentially to compare results. Wavefront errors are measured directly from the sample before and after correction. The image sequence with a frame rate of 30 seconds was achieved by the maximum intensity projection in each time period. A single frame without and with correction is shown in Figure 2.15(a). It shows a significant improvement in contrast and resolution. Without correction, the measured wavefront shows a dynamic change during the imaging time. The variation of the wavefront can be seen from the coefficient value change of different Zernike modes as shown in Figure 2.15(b). The short term data with 20 minutes imaging time is dominated by measurement noise from the wavefront sensor due to the low-level of photon emission. The enlarged PSF, approximately 1.8 μm, makes it impossible to obtain high resolution images. After correction, the coefficient value for Zernike modes are all below 0.05 μm. The Strehl ratio increases to approximately 0.6 as shown in Figure 2.15(c). The AO compensates those dynamic aberrations and produces a near perfect PSF with a spot size of 0.22 μm as shown in Figure 2.15(d).

Figure 2.15. 4D imaging of cycle 13 fly embryos with EGFP-Cnn label at a depth of 80 μm. A single frame without and with correction of a video movie (a). The coefficient value changes for Zernike modes $Z_2^2$ (astigmatism x, dashed line) and $Z_3^3$ (trefoil y, solid line) with and without AO during 20 minutes (b). The Strehl ratio change with (blue) and without (red) AO during 20 minutes (c). PSF size change with (blue) and without (red) AO during 20 minutes (d).59

These results show that AO microscopy with wavefront sensing using a fluorescent protein guide star can correct for the dynamic aberrations induced by the ovoid shape of the Drosophila embryo at high resolution for live imaging. Compared to the use of a fluorescent microsphere as a reference guide star,28,29,30,31,32,33 using fluorescent protein labeled sub-cellular structures as a noninvasive method simplifies the tissue preparation
process and avoids the potential side effects of injecting fluorescent beads in live imaging. The guide star could be the same fluorescent protein used for imaging or a specially designed protein for use only as a guide star. A fluorescent protein with a long excitation wavelength such as td-Tomato with high quantum yield, less photo-bleaching and more stability will further extend the correction depth and provide a more robust wavefront correction for live imaging. Using fluorescent proteins with excitation wavelengths different from the imaging fluorescent proteins can avoid the need to switch the light path between the wavefront sensing and imaging paths. Of particular interest is the use of a centrosome as a guide star. We can always find one or two centrosomes per cell in all animal cells depending on their position within the cell cycle. It broadens the applications of the wavefront sensing method for different kinds of cells and sample types. Moreover, the size of the centrosome is approximately 1 μm, which is particularly suitable as a guide star for the SHWS. The centrosomes are usually located close to the nucleus. The separation of centrosomes in two cells is often large enough in three-dimensional space for wavefront measurement with less background noise and less influence from the neighboring guide stars.

In comparison with the image-based AO method, the wavefront sensing method used here requires less time for wavefront measurement, which is particularly suitable to correct the dynamic aberrations induced in live specimens. The total wavefront correction time is 600 ms which includes a 50 ms exposure time for the wavefront measurement, a 50 ms DM control time and a 500 ms flipper mirror switching time. The last one can be minimized to less than 1ms by using a fast steering mirror, or eliminated using a beam splitter. To further minimize the DM control time, open loop control to update the DM using the wavefront measurement and an accurate DM model can be applied after the calibration of the DM. In this case, the correction speeds would be only limited by the wavefront measurement. Applying Field Programmable Gate Arrays (FPGA) in wavefront measurement will be beneficial for time-critical applications. For tissues with small isoplanatic patches, the guide star searching algorithm can find the optimal local guide star in each patch. A larger field of view with correction can be provided by stitching those patches together or by using conjugate AO. Finally, the application of FPCGS also simplifies the design of the hardware and software. Due to sharing a similar concept of wavefront sensing based on a laser guide star in astronomy and vision science, the knowledge of AO application in those fields has facilitated its application in microscopy.

2.3.3 AO Two-Photon Microscopy

The ability to image intact tissues and living animals with high resolution and depth penetration makes two-photon microscopy an invaluable tool for studying structure and function of cellular constituents within scattering tissue. Compared with the visible light used in confocal microscopy, the near infrared light used in two-photon microscopy experiences less scattering in biological tissue. The light detection is more efficient since both ballistic and diffuse emission light is collected. However the penetration depth is still limited by scattering and optical aberration. The optical aberration is caused by the variation in refractive index from the inhomogeneous optical properties of the tissues and the refractive index mismatch between the tissue and mounting medium. Most AO two-photon microscopes (AO-TPM) are based on indirect methods of aberration measurement which utilize processing of the final image.
extended exposure time for indirect methods can cause photo-bleaching and may limit the bandwidth for live imaging. To increase the wavefront correction speed, wavefront measurement is a promising method for dynamic in-vivo imaging applications. Coherence-gated wavefront sensing (CGWS) has been used for measuring the wavefront. This approach is based on backscattered light rather than fluorescent light. This approach requires a complicated interferometric arrangement, but it does eliminate the need for a Shack-Hartmann wavefront sensor. An alternative method for adaptive correction in two-photon microscopy used reflected light and confocal imaging for depth selection. An alternative method for adaptive correction in two-photon microscopy used reflected light and confocal imaging for depth selection. This approach simplifies the optics in the wavefront detection light path relative to interferometry and reduces the potential of inducing additional aberrations. Researchers utilized a Shack-Hartmann wavefront sensor that allowed the wavefront to be measured by acquiring a single image, improving the bandwidth of the AO feedback loop. Another approach uses pupil segmentation, however this approach is too slow for dynamic live imaging of events that change on timescales of seconds. This approach also used small fluorescent beads as references for measuring image shifts. Improvements to this approach using multiplexed aberration measurement have increased the speed and accuracy for discontinuous wavefronts.

2.3.3.1 Non-Linear Guide Star

Direct wavefront measurement with a Shack-Hartmann wavefront sensor and a non-linear guide star using two photon excitation has been demonstrated for correcting aberrations in two-photon microscopy. An advantage of a two-photon guide star is that light is confined to just the focal region by the non-linear excitation process, eliminating the need for a pinhole to eliminate out of focus fluorescence that occurs for a one-photon guide star. However, the long 800 ms integration time for making wavefront measurements is too slow for dynamic in-vivo imaging. A faster approach (30 ms) uses auto-fluorescent guide stars. As a label-free method, no special sample preparation is required. Auto-fluorescence from retinal lipofuscin by one-photon excitation has been used as a linear guide star to measure the wavefront of the human-eye in vision science. Auto-fluorescence can also be used in dynamic in-vivo biological microscopy by combining it with two-photon excitation, where only the fluorophores at the focus plane are excited for generation of a guide star with minimal background noise. Here the intrinsic fluorophores are illuminated by a near infrared ultrashort pulsed laser with an appropriate wavelength, which is often different from the excitation wavelength of common fluorescent proteins such as green fluorescent protein (GFP) and red fluorescent protein (RFP). This induces less photo-bleaching to the labeled fluorescent proteins during wavefront sensing. This method is particularly effective when the fluorescence from fluorescent proteins is too weak for wavefront sensing. After wavefront measurement, the measured wavefront error can be compensated by a deformable mirror using open loop control. High-speed and high-performance wavefront measurement and correction are the critical advantages of wavefront measurement over other sensorless adaptive optical systems. This method is used for live imaging of a Drosophila embryo labeled with (GFP) and (RFP), enabling measurements of aberrations in the middle of a Drosophila embryo. The layout of the AO-TPM is shown in Figure 2.16. Two-photon excitation is generated by a tunable (680-1080 nm) mode locked Ti:Sapphire laser (140 fs, 80 MHz, Chameleon Ultra II, Coherent). The intensity of the laser is modulated by an electro-optic modulator (model 350-80LA, Conoptics Inc.). A 60x water immersion objective with a numerical
aperture of 1.2 was used (Olympus Microscope, Center Valley, PA) for imaging. In order to correct the wavefront, a deformable mirror (DM) (Boston Micromachines) with 140 actuators and 3.5 μm of stroke is placed in the optical path, where it is conjugate to the exit pupil of the objective, the X and Y scanners and the wavefront sensor. The optical system includes three telescope relay sub-systems. Lenses L1 and L2 image the exit pupil of the objective lens onto the X scanner. They de-magnify the pupil from 7.2 mm to 4 mm. Lenses L3 and L4 relay the X scanner conjugate to the Y scanner. This design minimizes the movement of the scanning beam at the exit pupil of the objective lens and the emission light at the DM, which is important for accurate wavefront measurement and correction. Lenses L2 and L4 also serve as scanning lenses. The current design is optimized for an optical scanning angle of 4.4 degree, which provides a field of view of 128 μm on the sample with a 60x objective. Lenses L5, L6 and L7 image the pupil of the Y scanner onto the DM. For non-descanned detection, two photomultiplier tubes (PMT) (H7422-20 and H7421-40, Hamamatsu) and lenses L8, L9 are located immediately after the objective lens, which collects the emitted light. The emission light is separated by single edge dichroic beam splitters (FF705-Di01, Semrock) for dual-color imaging. During wavefront measurement, two-photon images are captured first. A guide star searching algorithm detects the best guide star based on the intensity and location from the image. Then two galvo scanners (6215H, Cambridge Technology) steer the beam to the best location in the sample to generate a guide star. The Shack-Hartmann wavefront sensor (SHWS) with a 44×44 element lenslet array (AOA Inc., Cambridge, MA) and an electron multiplying CCD camera (Photometrics) utilizes the emission light from the sample for wavefront measurement. Because the SHWS and DM are located on the emission and excitation paths, respectively, open-loop DM control is applied. This configuration will eliminate additional dichroic mirrors and switchable mounts for closed loop configuration, where the SHWS should be located behind the DM. It is also possible to locate the SHWS in a de-scanned position behind the scanners to enable averaging of the wavefront measurements.

In this system, a mathematical model for an accurate open-loop control of the deformable mirror is applied. To achieve open-loop control of the DM, an accurate model and calibration procedure are required. In our system, we apply mathematical modeling of the mirror surface based on the thin plate equation as follows,

\[ \nabla^4 \omega(r) = \frac{f_p(r)}{D} \]

where \( f_p(r) \) is the plate force, \( \omega \) is the displacement of the plate, \( D \) is the flexural rigidity of the plate. The plate force is the sum of spring forces \( f_s(\omega) \) and electrostatic forces \( f_E(v, \omega) \), where \( v \) denotes voltage applied on the actuator. \( f_s(\omega) \) and \( f_E(v, \omega) \) can be calculated from displacement measurement during calibration. Accurate look-up tables of these two parameters for different \( v \) and \( \omega \) are generated. During the system operation, the desired wavefront for compensation of the tissue induced aberration is measured by the SHWS. Then the plate force \( f_p(r) \) is calculated using Equation 8 and \( f_s(\omega) \) is retrieved from look-up tables based on the desired displacement \( \omega \) of the mirror surface. Finally the desired voltage \( v \) on the DM is estimated from the loop-up table \( f_E(v, \omega) \). A Zygo interferometer was used to measure the surface displacement during the calibration. The root mean square (RMS) error of the open loop control is around 17 nm for a DM displacement of 500 nm.

2.3.3.2 Results

To make an accurate wavefront measurement using the SHWS, the guide star should be smaller than the diffraction limit of the wavefront sensor. Also the number of photons coming from the guide star should be high enough to provide the required signal-to-noise ratio for the wavefront measurement. Thanks to the nonlinear excitation, the fluorescent emission is localized to a small area, which is often small enough to use as a guide star. However, the intensity of the guide star depends on the distribution of the fluorescently-labeled structure. An example shown in Figure 2.17 is the two-photon image of a Drosophila embryo labeled with GFP and RFP excited by a laser with different wavelengths at a depth of 51 \( \mu \)m. The laser power at the sample is 17.5 mW. At 1000 nm excitation, most of the RFP labeled nuclei structures can be observed near the membrane. In the middle of the embryo, because the yolk induces a large amount of aberration and scattering, the intensity of the nuclei structures becomes much dimmer. At 920 nm excitation, GFP labeled centrosomes are also seen to be distributed near the membrane as shown in Figure 2.17(b). It is extremely difficult to use this fluorescence as a guide star in the middle of the embryo. Unfortunately, the mitosis process early in the development of the embryo (first 9 cycles) occurs at the middle of the embryo, where the first cycles lasts for 10 min and begins once the egg has been laid. High-resolution and high-speed imaging of this process are critical for this study. In the Drosophila embryo, a major source of auto-fluorescence is from yolk granules and the vitelline membrane as shown in Figure 2.17(c). Although the yolk does not contain any RFP or GFP, auto-fluorescence arises from endogenous fluorophores with emission spectra similar to that of nicotinamide adenine dinucleotide (NADH). At 800 nm excitation wavelength, the fluorescence from the yolk is even brighter than the RFP. Because auto-fluorescence is a natural emission from biological structures, it exists even before the first mitosis cycle. This special feature makes it particularly suitable as a guide star for imaging the embryo at the early stages. Figure 2.17(d) shows the wavefront measurement at a depth of 51\( \mu \)m.
using auto-fluorescence for the guide star. During wavefront measurement, the laser is parked on the fluorophores indicated by the arrow. The laser power at the sample is 30 mW. The exposure time for a single measurement is 30 ms. The RMS error of the wavefront is 0.387 μm.

Figure 2.17. Two-photon images of a Drosophila embryo at a depth of 51 μm with excitation wavelengths of 1000 nm (a), 920 nm (b) and 800 nm (c). The wavefront (d) at the middle of embryo is measured by a SHWS in 30 ms using an auto-fluorescent guide star. During wavefront sensing, the laser illuminates the intrinsic fluorophores indicated by an arrow shown in (c). The scale bars are 10 μm.

The two-photon images of yolk auto-fluorescence at a depth of 51 μm before and after correction are shown in Figures 2.18(a) and 2.18(b). Each image is the maximum intensity projection of the three consecutive focal planes (1 plane/μm), which takes 6 seconds to achieve the whole stack. Because of the fast motion of the yolk during this imaging time, there is a small variation in structures shown in these two images. The excitation wavelength is set at 800 nm for maximizing the yolk auto-fluorescence. The structures of the endogenous fluorophores in yolk are much clearer after correction, and the noise is reduced dramatically. A two-photon image of RFP labeled nuclei structure without correction is shown in Figure 2.18(c). Compared with the yolk auto-fluorescence, less RFP labeled structures can be selected for use as the guide star and the intensity of the fluorescence is much lower. Figure 2.18(d) shows the two-photon image after correction. The image after correction is much brighter than before correction. The intensity profile along the dashed lines across nuclei is shown in Figure 2.18(e). The peak intensity increased by 2x. The fast direct wavefront measurement is particularly suitable to correct dynamic aberrations induced by live specimens. To validate this advantage, time-lapse wavefront aberrations were measured at a depth of 50 μm with a time resolution of 10 seconds. The RMS wavefront changed by around 0.1 λ in the first 10 seconds. The fast wavefront measurement will benefit the correction of these dynamic aberrations.
Figure 2.18. Two-photon imaging of a live Drosophila embryo at a depth of 51 µm. The images of yolk auto-fluorescence before (a) and after correction (b). The RFP labeled nuclei structure before (c) and after (d) correction. The intensity profiles (e) along lines in (c) and (d). The scale bars are 10 µm.88

2.3.4 Imaging Deeper

Since Shack-Hartman wavefront sensing depends on ballistic light, it will be limited in depth by scattering. This approach can be extended to tissues that strongly scatter visible light by exploiting the reduced scattering of near-infrared guide stars. This method enables in vivo two-photon morphological and functional imaging as deep as 700 µm inside the mouse brain.67 Another approach uses feedback-based wavefront shaping to focus light onto a guide star through scattering tissue.95 With feedback-based wavefront shaping for focusing light onto the guide star the signal-to-noise ratio and the RMS wavefront error of the laser guide star through scattering tissue can be more than doubled, potentially extending the imaging depth for AO microscopy, as shown in Figure 2.19.
2.4 Conclusions & Future Directions

In this chapter we have reviewed the use of direct wavefront sensing for AO in biological imaging including wide-field, confocal and multiphoton microscopy. Adaptive optical microscopy using direct wavefront sensing has been used to increase the resolution and signal intensity. Some of the benefits for this approach are faster correction for live imaging, lower-light exposure and less photo-bleaching since the wavefront sensing is accomplished in a single measurement rather than iterative measurements required for sensorless approaches.\textsuperscript{96,97,98} An additional benefit for using direct wavefront sensing is that the wavefront aberration is directly measured and corrected rather than optimized. The objective is getting as close as possible to the diffraction limit of the optical system, as measured by the Strehl ratio, rather than optimization of the overall image brightness or sharpness. The cost for these benefits is increased system complexity due to the requirements for the formation of a guide star in wide-field and confocal imaging, and the need for a wavefront sensing system such as a Shack-Hartmann wavefront sensor or an interferometer. In multiphoton microscopy a point guide star is automatically formed at the focus. Future work will most likely combine AO with higher-order wavefront shaping as imaging proceeds deeper into the sample where both refraction and scattering limit the imaging resolution, intensity and depth.

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