Adaptive optics two photon microscopy with direct wavefront sensing using autofluorescent guide-stars
Xiaodong Tao, Andrew Norton, Matthew Kissel, Oscar Azucena and Joel Kubby
Jack Baskin School of Engineering, Univ. of California, Santa Cruz, 1156 High St., Santa Cruz, CA, USA 95064

ABSTRACT
A fast direct wavefront sensing method for dynamic in-vivo adaptive optical two photon microscopy has demonstrated. By using the direct wavefront sensing and open loop control, the system provides high-speed wavefront measurement and correction. To measure the wavefront in the middle of a Drosophila embryo at early stages, autofluorescence from endogenous fluorophores in the yolk were used as reference guide-stars. This method does not rely on fluorescently labeled proteins as guide-stars, which can simplify the sample preparation for wavefront measurement. The method was tested through live imaging of a Drosophila embryo. The aberration in the middle of the embryo was measured directly for the first time. After correction, both contrast and signal intensity of the structure in the middle of the embryo was improved.

Keywords: Adaptive optics, two photon microscopy, guide-star, autofluorescence

1. INTRODUCTION
Two photon microscopy has became an invaluable tool for studying structure and function of cellular constituents within scattering tissue because of its ability to image intact tissues and living animals with high resolution and depth penetration. The near infrared beams used in two photon microscopy experience less scattering from biological tissue. Furthermore the non-descanned photon detection is more efficient where both ballistic and diffuse emission light is collected [1]. However scattering and optical aberration are still two important issues to limit the penetration depth. 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. To overcome this issue, adaptive optics (AO) has been successfully applied in microscopy [2]. The easiest way to integrate AO into a microscope is based on indirect sensorless optimization methods which use information from a sequence of images to estimate the aberration [3,4,5,6]. However the extended exposure time causes photo-bleaching and limits the bandwidth for live imaging. To increase the wavefront correction speed, direct wavefront measurement becomes a promising method for dynamic in-vivo imaging applications. In order to measure the wavefront error, wavefront sensors often require a “guide-star”, which is a reference point-source behind the inhomogeneous medium for measuring the shape of the wavefront. Injected microspheres and fluorescent proteins have been applied in wide-field and confocal microscopes as guide-stars [7,8,9]. A non-linear guide-star using two photon excitation has been demonstrated for correcting coupling and focusing aberrations in two photon microscopy [10], however their long integration time for making wavefront measurements is too slow for the dynamic in-vivo imaging we require.

In this paper we demonstrate a fast direct wavefront measurement method in an AO two photon microscope (AOTPM) using autofluorescent guide-stars. Because the autofluorescence is from the endogenous fluorophores in the sample, no special sample preparation is required. Autofluorescence 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 [11]. Here we demonstrate it in a two photon microscope, where only the fluorophores at the focus plane are excited for generation of a guide-star with minimal background noise. The intrinsic fluorophores are illuminated by a near infrared ultrashort pulsed laser with appropriate wavelength, which is often different from the excitation wavelength of fluorescent proteins such as green fluorescent protein (GFP) and red fluorescent protein (RFP) [12]. The wavefront sensing induces less photobleaching to the labeled fluorescent proteins. This method is also well suited when the fluorescence from fluorescent proteins is too weak for wavefront sensing. After wavefront measurement, the measured wavefront error was compensated by a deformable mirror using an open loop control [13]. High-speed and high-performance wavefront measurement and
correction are the critical advantages of direct wavefront measurement over other sensor-less adaptive optical systems. The proposed method was tested through live imaging of a Drosophila embryo labeled with (GFP) and (RFP).

2. METHOD

2.1 System setup

Figure 1 shows the layout of the AOTPM. The system was designed and optimized using the optical design software CODE V. The 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 demagnify the pupil from 7.2 mm to 4mm. Lenses L3 and L4 relay the X scanner conjugate to the Y scanner. Lens group L5, L6 and L7 relays the pupil plane onto a DM. 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 degrees, which provides a field of view of 128 μm on the sample with a 60x objective. The photomultiplier tube (PMT) (H7422-20 and H7421-40, Hamamatsu) is configured as a non-descanned photon detection and lenses L8, L9 are located immediately after the objective lens, which collects the emitted light. 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 [14]. 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.

![Figure 1](image_url)

2.2 Open-loop control of the DM

Because the SHWS and DM are located on the emission and excitation paths respectively, open-loop DM control is applied. An accurate model and calibration procedure are required for open-loop control. In our system, we apply mathematical modeling of the mirror surface based on the thin plate equation as follows [13],

\[ \nabla^4 \omega(r) = f_p(r)/D \]  

where \( f_p(r) \) is the plate force, \( \omega \) is the displacement of the plate. The plate force is the sum of spring forces \( f_s(\omega) \) and electrostatic forces \( f_e(\nu, \omega) \), where \( \nu \) denotes voltage applied on the actuator. \( f_s(\omega) \) and \( f_e(\nu, \omega) \) can be calculated from displacement measurement during calibration. Accurate look-up tables of these two parameters for different \( \nu \) and \( \omega \) are generated [13]. 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 1 and \( f_s(\omega) \) is retrieved from look-up tables based on the desired displacement \( \omega \) of the mirror surface. Finally the desired voltage \( \nu \) on the DM is estimated from the loop-up table \( f_e(\nu, \omega) \). 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 17nm for amplitude of 500nm.

2.3 Autofluorescent guide-stars

Two important factors are involved in an accurate wavefront measurement. First the guide-star should be smaller than the diffraction limit of the wavefront sensor. Second there needs to be enough photons coming from the guide-star to provide the required signal to noise ratio for the wavefront measurement. Due to the nonlinear excitation, the fluorescent emission is localized to a small restricted area, which is often small enough to use as a guide-star [10]. However the intensity of the guide-star depends on the spatial distribution of the fluorescently labeled structure. An example shown in Figure 2 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. Most of the RFP labeled nuclei structure excited at the wavelength of 1000nm can be observed near the membrane. In the middle of the embryo, because the yolk induces large amounts of aberration and scattering, the intensity of the nuclei structure becomes much dimmer. GFP labeled centrosomes at 920nm excitation are seen to be distributed near the membrane as shown in Figure 2 (b). 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 [15]. High resolution and high speed imaging of this process are critical for this study. However the concentration of the fluorescent protein at the middle of the embryo is not high enough to emit enough photons for an accurate wavefront measurement. An alternative guide-star using autofluorescence from yolk granules [16] can be a good candidate for wavefront sensing. Although the yolk does not contain any RFP or GFP, autofluorescence arises from endogenous fluorophores with emission spectra similar to that of NADH [17]. At 800nm excitation wavelength, the fluorescence from the yolk is even brighter than the RFP as shown in Figure 2(c). Because autofluorescence is a natural emission from the 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 (d) shows the wavefront measurement at a depth of 51\( \mu \)m below the coverslip using autofluorescence 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 30mw. The exposure time for a single measurement is 30ms, that is over 25 times faster than the previous work using a non-linear guide-star [10]. The RMS error of the wavefront is 0.387\( \mu \)m.
Figure 2. Two photon images of a Drosophila embryo at a depth of 51µm below the coverslip with excitation wavelengths of 1000nm (a), 920nm (b) and 800nm (c). The wavefront (d) at the middle of embryo is measured by a SHWS in 30ms using an autofluorescent guide-star. During wavefront sensing, the laser illuminates the intrinsic fluorophores indicated by an arrow shown in (c). The scale bars are 10µm.

3. EXPERIMENTAL RESULT

To test the proposed method, we first captured two photon images of yolk autofluorescence at a depth of 51µm before and after correction as shown in Figures 3 (a) and (b). The excitation wavelength is set at 800nm for maximizing the yolk autofluorescence. Each image is the maximum intensity projection of the three consecutive focal planes (1 plane/um), which takes 6 seconds to achieve the whole stacks. The contrast of structures of the endogenous fluorophores in yolk is improved after correction, and the noise is reduced dramatically. Then we tune the wavelength to 1000nm and achieve the two photon images of RFP labeled nuclei structure without correction as shown in Figure 3 (c). Compared with the yolk autofluorescence, less RFP can be selected for use as guide-star and the intensity of the fluorescence is much lower. Figure 3(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 3 (e). The peak intensity increased by 2x.

The ability to correct aberrations and dynamically image features deep within living organisms is critical for studying cellular dynamics, motility, and cell and tissue morphology. The direct wavefront sensing method is particularly suited in this situation. To validate this advantage, time-lapse wavefront aberrations were measured at a depth of 50m with a time resolution of 10s. Figure 4 shows the RMS wavefront changed by around 0.1 λ in the first 10 sec. Therefore statistic or slow wavefront measurement and correction would not be able to follow the dynamic wavefront change. Fast wavefront measurement will definitely benefit the correction of these dynamic aberrations.
Figure 3. Two photon imaging of a live Drosophila embryo at a depth of 51 µm below the coverslip. The images of yolk autofluorescence before (a) and after correction. The RFP labeled nuclei structure before (a) and after (b) correction. The intensity profiles (c) along lines in (c, d) before (blue) and after (red) correction. The scale bars are 10 µm.

Figure 4. RMS wavefront change during 300 sec with a time resolution of 10 sec (Media 1). The phase maps of the wavefront change at 30 sec, 50 sec and 150 sec are also shown.

4. CONCLUSION

In this paper, an adaptive optical two photon microscope using direct wavefront sensing is demonstrated. Using autofluorescence from endogenous fluorophores as reference guide-stars makes it possible to correct the aberration in the middle of the embryo. As a non-invasive method, the autofluorescent guide-star allows fast wavefront measurements in the middle of an embryo at the very early dynamic stages. The experimental results show its potential to image the early mitosis process of the Drosophila embryo in vivo. Using intrinsic fluorophores as guide-stars will benefit a wide range of
label-free nonlinear microscopes, such as Second Harmonic Generation (SHG), Third Harmonic Generation (THG) and Coherent Anti-Stokes Raman Scattering (CARS) microscopes, which will be investigated in our future research. By using synchronized pulses from a femtosecond laser and an optical parametric oscillator [18] with different wavelengths, the wavefront sensing and two photon imaging can operate simultaneously.

ACKNOWLEDGMENTS

The results presented herein were obtained at the W.M. Keck Center for Adaptive Optical Microscopy (CfAOM) at University of California Santa Cruz. The CfAOM was made possible by the generous financial support of the W.M. Keck Foundation. We would like to thank Prof. William Sullivan, Dr. Shaila Kotadia and Dr. Frederic Landmann of MCD Biology, UC Santa Cruz, for the Drosophila embryo samples and useful conversations. We also acknowledge the technical support from Ben Abrams in the UCSC Life Sciences Microscopy Center.

REFERENCES