# Adaptive optics two-photon fluorescence microscopy

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## Abstract

Non-linear imaging is widely used in biological imaging, primarily because of its ability to image through tissue to depth of a few hundred micrometers.  Because two photons need to be absorbed to excite a fluorophore in this instrument, the probability of fluorescence emission of a detectable photon scales with the intensity squared of the beam. As a result, aberrations in the beam path that reduce the peak intensity of the focused, scanned laser spot have a significant effect on the instrument performance. Methods for reducing those aberrations should allow higher resolution and detection sensitivity, and deeper tissue imaging.


In this paper, I will describe a non-linear imaging microscope that has an adaptive optics (AO) subsystem to compensate for beam path aberrations. The AO system relies on a 140 actuator deformable mirror, controlled using a stochastic gradient descent algorithm with feedback from a fluorescence sensor. The controlled instrument will be used for *in vivo* imaging of mouse skin, lymph nodes, and skull bone marrow at depths up to 500µm.

**Key words:**  adaptive optics and deformable mirror

## 1. INTRODUCTION

Non-linear imaging provides an intrinsic three dimensional view of the imaging target [1]. Since fluorophore excitation increases quadratically (e.g. two-photon excitation) with the value of the illumination light intensity, the excitation has a smaller imaging volume laterally and axially comparing to single photon excitation.

The non-linear imaging microscope has been used widely for *in vivo* fluorescence imaging deep into tissue. The specific application corresponding to this paper is for imaging bone marrow environment to study tumor cell metastasis and stem cells [2].


Optical scattering, absorption and aberrations are the major factors limiting the achievable imaging depth. Optical aberrations distort the light wavefront in the beam path, preventing

formation of diffraction limited focal spot. The size of the focal spot during imaging is critical since a high photon density is desired for the non-linear fluorescence excitation. This is particularly attractive for multiple-photon microscopy due to the nonlinear excitation mechanism. For example, for a second harmonic excitation, a small improvement in the focal spot diameter (r) can result in a large improvement in signal intensity (goes up as $r^4$)

The sources of aberrations are from both the microscopic objective and imaging targets. The imaging target itself brings intrinsic optical aberration due to inhomogeneous optical index of refraction, and the aberration intends getting larger with a deeper imaging depth. The combination of high numeric aperture objective, objective immersion media, cover glass and imaging target degrades beam quality significantly due to different optical refractive index in each media, e.g., spherical aberration.

With increasing the light wavelength from light source, tissue scattering is becoming less significant, but chromatic aberration from microscopic objective is becoming a more significant factor to limit the imaging depth. For example, current commercial microscopic objective at 1300nm does not exist, which is being used in our experiment setup.

Several groups have conducted research on wavefront correction related to multiphoton scanning microscopy for (*in vitro*) imaging [3-6]. Here we are presenting a non-linear fluorescence microscope with AO for (*in vivo*) imaging with a dynamic correction on wavefront up to 7th order Zernike polynomial.

Conventional AO systems employ a sensor to measure wavefront errors in the image-path of the optical system. These measured wavefront errors can be used as feedback to the wavefront corrector in a closed control loop that compensates for aberrations. However, in the fluorescence microscope the excited photons are not collected in an imaging beam, so conventional wavefront sensing is not practical. This is a fundamental problem for AO control, which requires some measured or modeled wavefront distortion data as a basis for compensation.

One alternative is to use a scalar measure of fluorescence intensity itself as the controller feedback signal. In this approach, the controller objective is to maximize fluorescence intensity by controlling the multi-degree-of-freedom deformable mirror. The approach (known as sensorless adaptive optics) is inefficient in comparison to conventional AO, but has proven effective for complex AO applications in which wavefront sensors are impractical [7].


## 2. METHODS


2.1 Stochastic Parallel Gradient Descent Algorithm


The stochastic parallel gradient descent algorithm maximizes the metric signal by perturbing the mirror shape, then assessing the effect on the desired metric (e.g. fluorescence signal) and using this as the basis for iterative convergence toward an optimal (or locally optimal) shape. First, all actuators (u) in the deformable mirror are perturbed by a unit amount (du) in a direction that is randomly signed for each actuator (+/- i). The fluorescence signal is measured, and stored as the metric value (J+). Then the mirror actuators are perturbed in the opposite direction (-i), and the metric is recorded again (J-). The differences between the positive and negative perturbations provide a gradient in the multidimensional actuator space, and a new state for the deformable mirror is determined by multiplying each actuator's signed perturbation by the global gradient and adding this value to the as an offset to the actuator state.

A Zernike polynomial function of one of a complete set of functions is defined and orthogonal over the unit circle [8]. A set of Zernike polynomials can used to represent the mirror surface shape, which is matched to pupil plane in the microscope setup. In stead of perturbing each actuator in the stochastic parallel gradient descent algorithm, we can change DM shape accordingly by perturbing a set of Zernike coefficients simultaneously.

Zernike polynomials have been widely used as a set of orthogonal wave functions to characterize wavefront aberration in an optical setup. Some radial symmetric terms (e.g. defocus and spherical) are well matching the wavefront aberrations in any microscopy caused by index mismatching in the imaging. A proper set of Zernike polynomial coefficients can be chosen to improve the loop efficiency based on the known information of the optics.

The MEMS DM used in this work is produced by Boston University and Boston Micromachines Corporation. The DM includes 140 actuators and has 4.4mm clear aperture and good. In a previous paper, we have demonstrated that the first 7th order Zernike polynomial shape can be produced by this DM in open loop fashion [9]. Figure 1 shows Optical microphotograph of the MEMS DM and a drawing of the device concept.
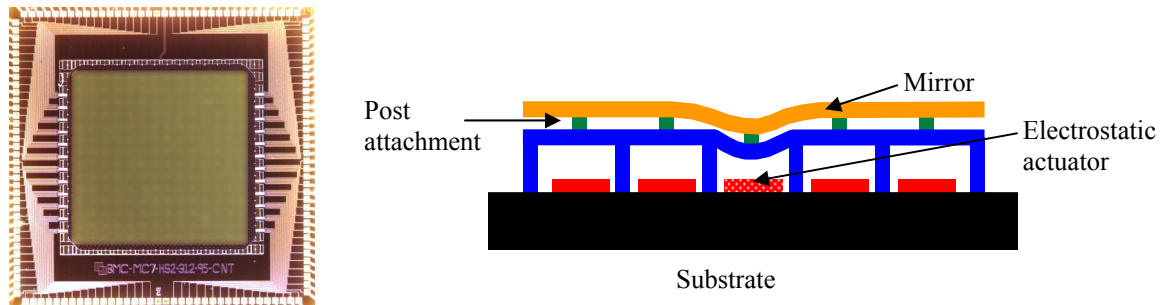


Figure 1. Optical microphotograph (left) and cross sectional schematic (right) of the BMC MEMS DM. The clear aperture of the mirror is 4.4mm. It is supported on an array of 12 x 12 electrostatic actuators on a 400μm square pitch. The four corner actuators are not used. Each actuator is connected to the continuous mirror membrane by a single post attachment. Actuators can pull the mirror toward the substrate by up to 4μm in response to an applied voltage. Each actuator is controlled independently.

Gradient descent AO has inherent advantages when applied to biomedical microscopy. First, it uses as a control feedback the specific signal that is to be optimized. The goal for this AO system is to improve the fluorescence excitation in deep tissue. Using the light intensity itself as feedback signal for AO is a straightforward approach to that goal. Second, it decreases the complexity of the instrument setup by eliminating the need for reimaging and wavefront sensing, thereby improving the optical throughput and reducing cost.

2.2 Optical Setup

A schematic of the non-linear imaging microscope with AO is shown on figure 2. The setup occupies about a 0.5 m by 0.5 m area on an optical table. The apparatus includes five main components: light delivery, raster scanning, light detection and wavefront correction.
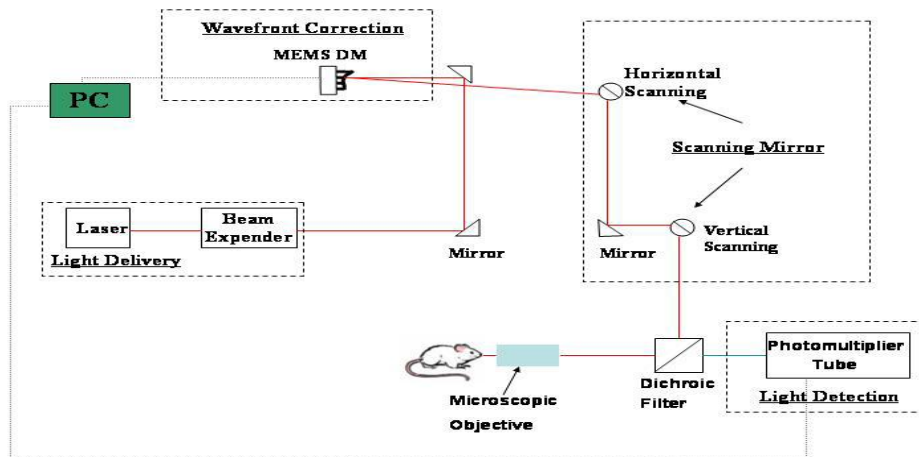
Figure 2. Adaptive optics two-photon fluorescence microscopy includes five main components: light delivery, raster scanning, light detection and wavefront correction.

Light is delivered from a solid state mode-locked Ti-sapphire laser and an OPO with combined tuning range from 700nm to 1500nm. The entrance pupil size is defined by the clear aperture of MEMS DM. The entrance pupil is relayed by lens pairs to the horizontal scanner, the vertical scanner and the microscopic objective. The two scanning mirrors work in a raster fashion, and creates 5x5 degree scanning field on the biological sample to form a two dimension image.

A dichroic filter is used to reflect the infrared light. The redirected infrared light is focused on the biological sample through a high NA microscopic objective. The (blue-shifted) fluorescence light passes through the dichroic filter and is detected by a photomultiplier tube.

## 3. Experiment and Result

To investigate the performance of AO setup, we used an artificial sample in the initial tryout. In this approach, we intentionally created a defocus by move the imaging target axially with certain amount of distance. The imaging target was a piece of paper with fluorescence marker. The image in Figure 3 with AO on shows a much stronger fluorescence excitation signal by correcting the defocus compared to the image with AO off.

## Defocus Correction



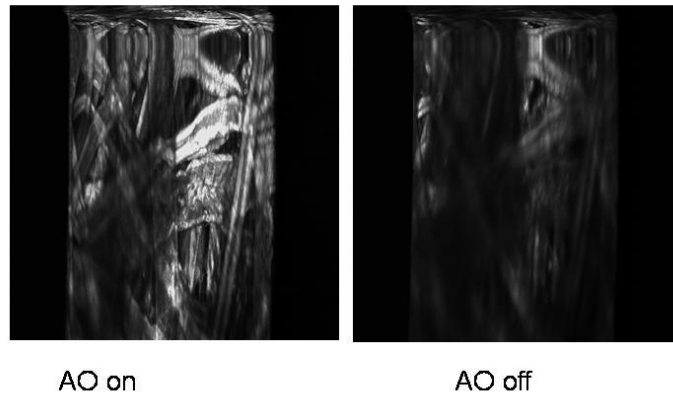AO on                    AO off

Figure 3.  Left: fluorescence image of paper fiber with AO on; Right: fluorescence image of paper fiber with AO off.

In Figure 4, the Zernike coefficient plot from final mirror shape shows a significant defocus term, that matches the designed aberration source. It is essentially proving the Zernike polynomial based gradient decent AO is effective on correcting wavefront aberration.
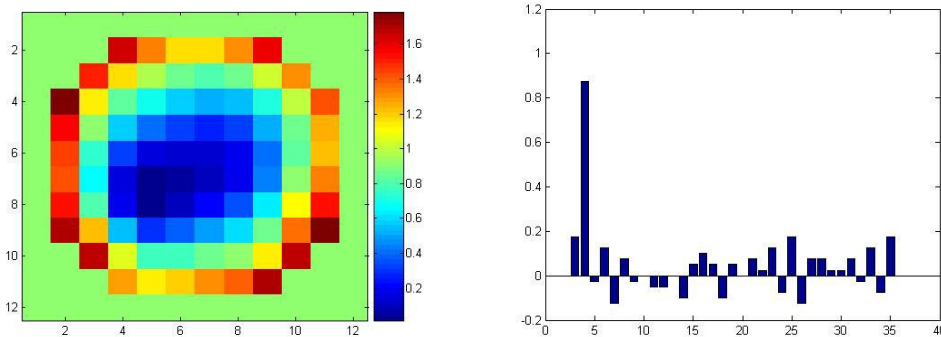


Figure 4.  Left: DM shape for defocus correction; Right: final: Zernike polynomial of DM shape.

Have demonstrated the effect of AO on an artificial sample, we moved to our main target. In this preliminary experiment, bone imaging of mouse skull was taken at 180µm into the bone. The illumination light was at 1300nm wavelength and a 0.9 NA, 30x, water immersion objective was used to image the mouse skull. All the fluorescence images were generated from second harmonic signal.  Clearly, bone cavity image in Figure 5 with AO on shows improved intensity and resolution.
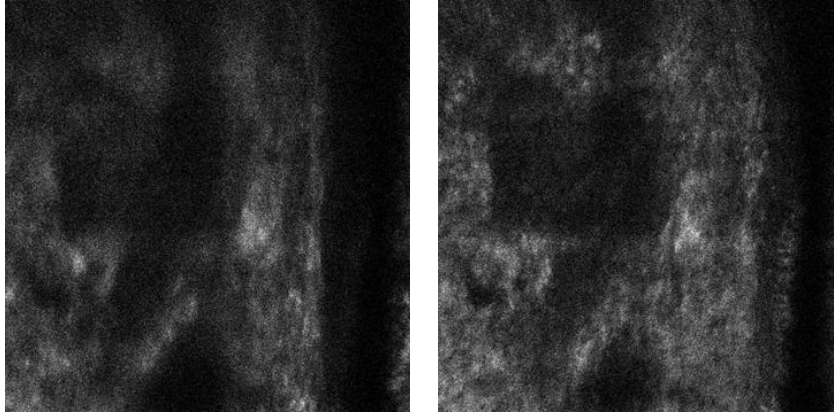
Figure 5.  Left: Bone cavity image in mouse skull with AO off; Right: cavity image in mouse skull with AO on.

## 4. CONCLUSION

In this work, we demonstrated stochastic parallel gradient descent AO is effective to correct aberration in the bone structure. Zernike basis is useful to characterize aberration source and can be used to improve AO efficiency. In the future work, bone imaging aberration will be characterized and bone marrow fluorescence imaging with AO will be tested.

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