# Two-Photon Differential Aberration Imaging using a Modulating Retroreflector Mirror

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**Abstract:** Differential Aberration Imaging (DAI) uses a deformable mirror to improve two-photon fluorescence microscopy by enhancing rejection of out-of-focus background from scattering. We report a new implementation of DAI using a Modulating Retroreflector Mirror.
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### Introduction

A major limitation to depth penetration when performing fluorescence imaging in thick tissue is caused by out-of-focus background. In standard confocal fluorescence microscopy, out-of-focus background is rejected by a pinhole; however, this rejection becomes ineffective at large depth penetrations because of background fluorescence scattering. Two-photon excited fluorescence (TPEF) microscopy [1] significantly outperforms standard confocal fluorescence microscopy in that it suppresses background generation (as opposed to rejecting background detection). Nevertheless, even TPEF microscopy suffers from a problem of out-of-focus background generation when imaging deep in thick tissue. In particular, out-of-focus background can arise from near-focus TPEF generated by weakly scattered excitation light or even from TPEF generated at the tissue surface where the ballistic excitation power density is non-negligible.

We have recently reported a simple solution to largely reject TPEF background in thick tissue called Differential Aberration Imaging (DAI) [2,3]. In this technique, we use a deformable mirror to intentionally introduce extraneous aberrations in the excitation beam path, which drastically reduces in-focus TPEF signal while leaving out-of-focus TPEF background largely unchanged. A simple subtraction of an aberrated from an unaberrated TPEF image then removes background while preserving signal.

Our previous results were achieved using a deformable mirror composed of 140 independently addressable elements, generously loaned to us by the Boston Micromachines Corporation (BMC). We have now obtained another type of deformable mirror produced by BMC called the Modulating Retroreflector Mirror (MRM). The MRM is essentially a sinusoidal grating pattern that modulates its amplitude with applied voltage. This device is much less expensive than the original deformable mirror and requires no specialized driver components. However, since our goal is to introduce destructive aberrations rather than to perform adaptive optical corrections, we are able to use the MRM to achieve DAI.

### Materials and Methods

The original overall setup for TPEF with DAI is very similar to a standard TPEF configuration, as shown in Figure 1.
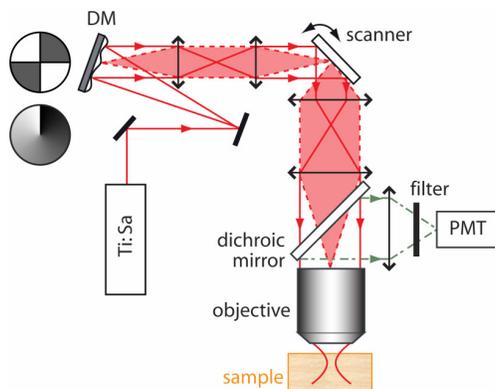
**NMD3.pdf**



Figure 1: Two-photon fluorescent imaging configuration with deformable mirror inserted to produce aberrations. From [3].

Our laser in the original implementation was a Ti:Sapphire, but is now a Ytterbium crystal type (Amplitude Systèmes "t-pulse", 1030 nm, 1.2W average power, 150 fs pulses).

The deformable mirror shown in Figure 1 is now the variable-amplitude sine grating MRM. The mirror has a single signal input that can range from 0 to 65V to vary the mirror surface from flat to a sine grating with a peak-to-peak distance of approximately 230 nm.

DAI requires two images: one normal TPEF image, and one with aberrations added. In a raster scanning system, we can take advantage of the "flyback" stroke – the part of the scan cycle when the scanning mirror returns to the edge of its range in preparation for another line scan – to acquire the aberrated image. Using the flyback time allows both the aberrated and normal images to be acquired without increasing the amount of time needed for a single normal image of the same size.

The aberrated image contains predominantly out-of-focus information, which is always low-resolution. Any high spatial frequency content present in the image can only be from noise. Therefore, it is advantageous to low-pass filter the aberrated image before subtracting it from the normal image.

**Results**

A key feature of an aberration applicable to our purpose is a decrease of signal in-focus and the preservation of out-of-focus background. Figure 3 demonstrates the fluorescent signal collected both with and without aberrations when a thin fluorescent plane is scanned through depth, and confirms the suitability of the MRM to DAI.
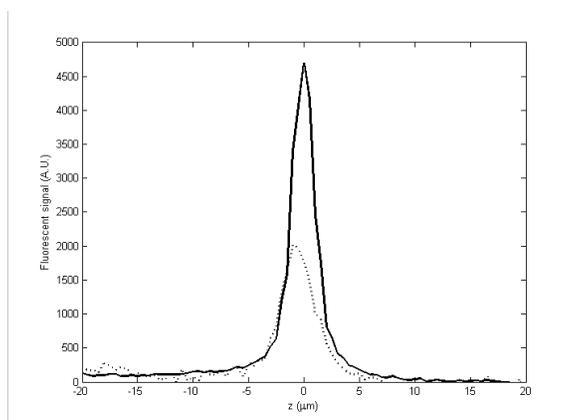


Figure 2: TPEF signal from thin plane, with aberrations (dotted) and without (solid), normalized to equal illumination power.

We can use DAI in any TPEF imaging situation when scattering causes an increase in out-of-focus background.  An example using a fluorescent pollen grain is shown in Figure 3.  After application of DAI (right), the out-of-focus fluorescence that permeates the center of the pollen grain in the normal TPEF image (left) is suppressed.
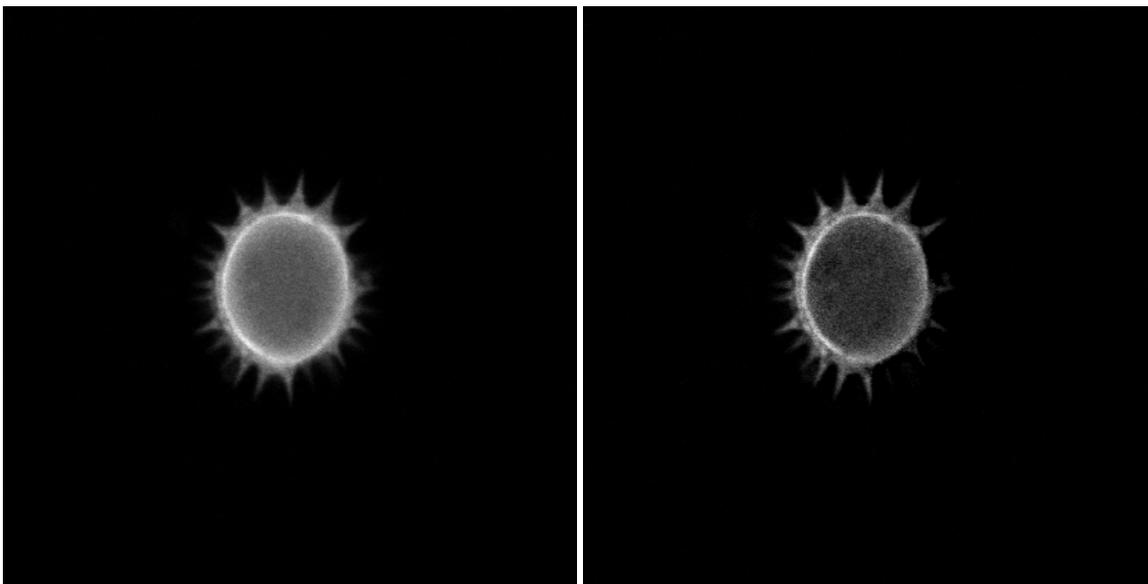


Figure 3: TPEF images of pollen grain.  FOV is approximately 110 μm square.  Left: Standard TPEF image. Right: DAI image using MRM.

**References**

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