

# Two-photon fluorescence microscopy with differential aberration imaging

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## ABSTRACT

We report our progress in the development of Differential Aberration Imaging (DAI), a technique that enhances two-photon fluorescence (TPEF) microscopy by improving rejection of out-of-focus background by means of a deformable mirror (DM). The DM is used to intentionally add aberrations to the imaging system, which causes dramatic losses to in-focus signal while preserving the bulk of the out-of-focus background. By taking the difference between TPEF images with and without added aberrations, the out-of-focus portion of the signal is further rejected. We now introduce an implementation of DAI using a new type of DM that can be produced at much lower cost.

**Keywords:** Two-photon fluorescence, deformable mirror

## 1. INTRODUCTION

Two-photon excitation fluorescence (TPEF) imaging [1] has become a mainstream imaging tool for biological imaging, particularly in scattering tissue [2]. TPEF is an attractive option for such applications due to its inherent ability to limit fluorescence emission to the small focal volume being probed. However, several factors limit the maximum depth to which TPEF can produce acceptable imaging performance.

Firstly, scattering media by definition cause significant loss to ballistic (unscattered) light. The amount of ballistic light that remains after passage through scattering tissue falls exponentially in relation to depth, causing a conjunctive increase in the requirement of illumination laser power. When scattering loss at a given depth is too severe to produce appreciable TPEF signal from the focal volume at maximum available laser power, obviously no TPEF imaging is possible.

Secondly, even in situations when even laser power exists to pass through enough ballistic light to generate two-photon fluorescence from the focus, the exponential relation between remaining power and depth means that the power at the surface can be quite high, generating its own two-photon fluorescence. As illumination power is increased in an effort to image at greater depths, the power density at the surface of the tissue rises exponentially, which can eventually lead to the domination of the in-focus signal by this surface-generated background [3-5].

Thirdly, at higher depths, the intensity of slightly scattered illumination near the focus also increases. This form of scattering, also known as “snakelike,” exhibits a higher probability of generating TPEF compared to diffuse multiple scattering [6,7]. Snakelike scattering causes unwanted excitation in a blurred halo of fluorescence surrounding the ballistic focal volume, and in conjunction with surface-generated background, further degrades image contrast.

We have developed Differential Aberration Imaging (DAI) [8,9] as a counter to the second and third limitations specified above. As in many adaptive optics implementations, we introduced a deformable mirror (DM) into the illumination path to dynamically shape the wavefront. However, instead of using the mirror to counteract aberrations in the tissue as such configurations are usually intended to do, we use the DM to intentionally cause extraneous aberrations. These aberrations cause significant distortion to the focal volume, resulting in a dramatic reduction in the amount of TPEF signal generated in-focus. On the other hand, the background fluorescence, having been generated relatively far from focus, remains relatively unaffected by the added aberrations. Therefore, by simply subtracting an image taken with extraneous aberrations from a normal TPEF image, we enhance the rejection of fluorescence generated from out-of-focus sources.

We have previously reported positive results using DAI to reject out-of-focus background. In this paper, we demonstrate an updated implementation of our technique. The principal change is in the DM used to introduce the extraneous aberrations. Known as a Modulating Retroreflector Mirror (MRM), this type of DM is available at a much lower cost than the model used in our earlier reports. The original DM as well as the MRM were produced and generously provided by the Boston Micromachines Corporation.

## 2. METHOD

Very few changes need to be made to a standard TPEF imaging setup to perform DAI. The DM is inserted into the illumination path, upstream of and conjugate to the scanning galvanometer mirrors (Figure 1). In our previous implementation, the DM was an array of 140 independently addressable elements, with which we projected aberrating patterns such as the ones illustrated in Figure 1. Since the DM is imaged to the scanning mirrors, it is in the Fourier plane of the sample.

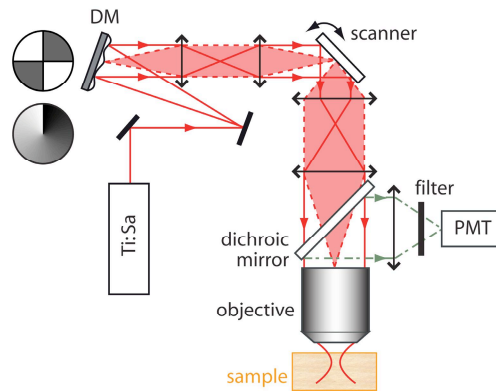


Fig. 1: TPEF setup with DM added for DAI. From [9].

We have replaced the full-fledged DM with the MRM, which is only capable of producing a sinusoidal phase profile, the amplitude of which is varied by its input voltage (0 to 65V). The period of the sine pattern is fixed at  $150\ \mu\text{m}$ . We found, however, that placing the DM in the Fourier plane as we had done previously did not produce a satisfactory aberration. The MRM in this case acts as a simple diffraction grating, spreading the beam into several diffraction orders when energized. Aperture limits in our system prevent all but the 0th and 1st orders from reaching the sample, resulting in three focal spots arranged on a line and equally spaced. With our laser wavelength and lenses, the distance between foci is approximately  $7\ \mu\text{m}$  with a 60x objective. At this spacing, the snakelike scattering halos from neighboring foci do not overlap significantly. As a result, the fluorescence from the snakelike halos falls as the aberration is activated, splitting the beams into the diffraction orders. Even though the fluorescence from the in-focus ballistic light also falls as a result of being split, the differential change between the in-focus and out-of-focus components is lost.

To decrease the spacing between the diffraction orders and to restore the overlap between neighboring snakelike scattering halos, the MRM was moved closer to the image plane instead of the Fourier plane. Complete alignment with the image plane is also undesirable, since the diffraction orders would simply reconverge to a single point in the focal plane, which would result in very little change to TPEF signal. By displacing the MRM slightly from the image plane, however, a small amount of separation between the diffraction orders is achieved, which meets our requirement of decreased in-focus signal while preserving the overlap between the scattering halos.

Differential measurements in general do suffer from a drawback: amplification of independent noise. Though DAI effectively reduces the mean contribution of out-of-focus background, the shot noise from the aberrated image is added to, not subtracted from, the shot noise of the unaberrated image, since the two sources of noise are uncorrelated. Fortunately, we are able to reduce the noise from the aberrated image by means of filtering. The aberrated image represents out-of-focus background, either from the surface of the tissue or the snakelike scattering halo. Both of these sources are large in size in comparison to the focal volume, and therefore represent low-resolution information. Any high-resolution content in the aberrated image must therefore be spurious, and likely resulting from noise rather than carrying any information about the sample structure. We can thus simply low-pass filter the aberrated image before subtracting it from the unaberrated image to prevent much of its shot noise from being added.

The low-frequency nature of the aberrated image also allows a time-saving shortcut. In a galvanometer mirror raster scanning system, there is usually a wasted “flyback” time when the line-scanning mirror returns from one side of the frame to the other in preparation for scanning the next line. The flyback is conducted more rapidly than the acquisition stroke to minimize wasted time. Since the aberrated image is limited to low-frequency content, it can be acquired during the flyback despite the large pixels that result from using the fast moving flyback beam. By taking advantage of time that would have been wasted, the acquisition time for the unaberrated and aberrated images combined is no longer than the time required for a single image.

### 3. RESULTS

#### 3.1 Uniform plane

A thin, uniform plane of fluorescent dye was imaged through a range of depths to verify the requirement that the in-focus signal changes significantly in comparison to out-of-focus signal with the activation of the MRM. The results are shown in Figure 2. The aberrated signal has been normalized to compensate for the loss of illumination power due to aperture limits when the mirror is active. At approximately 2.5  $\mu\text{m}$  from the focal plane in either direction, the unaberrated and aberrated profiles meet. The difference between the two essentially eliminates out-of-focus signal that originates from outside of 2.5  $\mu\text{m}$  from focus.

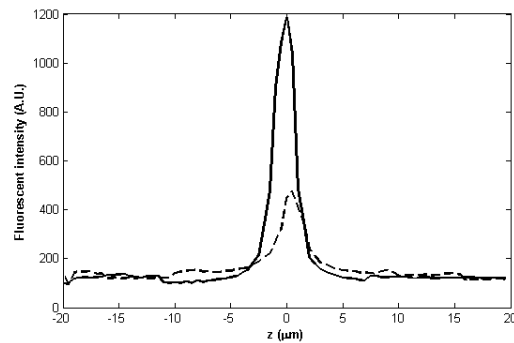


Fig. 2: Uniform fluorescent plane detected TPEF intensity, scanned through depth, unaberrated (solid) and aberrated (dashed).

#### 3.2 Effect on lateral resolution and the optical transfer function

We examined the point spread function and optical transfer function of both the unaberrated and aberrated cases. The TPEF image of a 1  $\mu\text{m}$  bead in both cases is presented in Figure 3. Since the aberration spreads the beam along only one axis, the image of the bead is distorted solely in that direction.

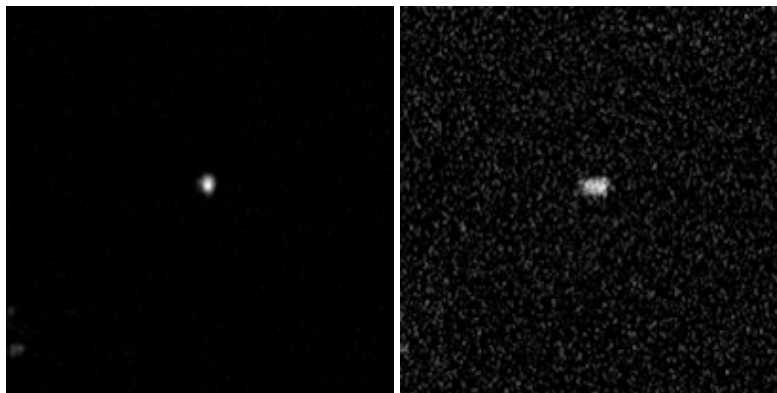


Fig. 3: TPEF image of 1  $\mu\text{m}$  bead without aberrations (left) and with aberrations (right), relative intensities not to scale.

A DAI image is formed by taking an image that contains both sharp, in-focus features, and subtracting from it an image that is blurred and out of focus. The result is a composite image that appears sharper, although without actually introducing higher supported spatial frequencies. This effect is best illustrated when considering the optical transfer function (OTF), the frequency domain representation of the point spread function. Figure 4 shows the magnitude of the numerically derived OTFs taken along the axis affected by the mirror modulation, both with and without aberrations, as well as the differential image. The differential case shows that the higher frequencies remain close to the unaberrated case, but their strength relative to the lower frequencies and total spectrum energy have increased. However, it is important to emphasize that DAI is not simply a 2D filtering algorithm. DAI is able to distinguish between out-of-focus and in-focus low frequency content.

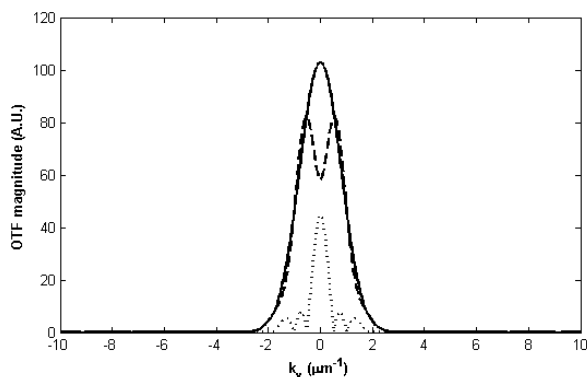


Fig. 4: Magnitude of numerically derived optical transfer function evaluated along axis of aberration. Unaberrated (solid), aberrated (dotted), and differential (dashed) cases shown.

### 3.3 Imaging results

DAI was used to image mouse brain hippocampus sections stained with sulforhodamine 101, a dye that preferentially stains astrocytes in neural tissue. Figure 5 compares imaging results using normal TPEF and DAI both with and without low-pass filtering the aberrated image. Both DAI cases show expected improvement in contrast and sharpness. The DAI image formed with the low-pass filtered version of the aberrated image shows superior shot noise levels without sacrificing resolution.

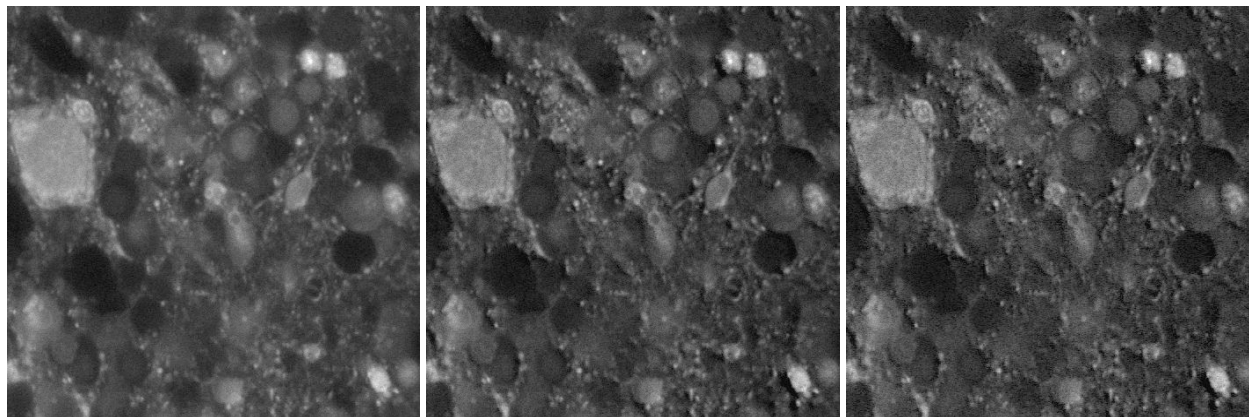


Fig. 5: Mouse brain hippocampus stained with sulforhodamine 101. Standard TPEF image (left), DAI images with (center) and without (right) low-pass filtering the intermediate aberrated image.

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