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Pozzi, Paolo; Wilding, Dean; Soloviev, Oleg; Vdovine, Gleb; Verhaegen, Michel

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Adaptive optics in digital micromirror based confocal microscopy

P. Pozzi[*a], D.Wilding[a], O.Soloviev[a,b], G.Vdovin[a,b], M.Verhaegen[a]
[a]Delft Center for Systems and Control, Delft University of Technology, Mekelweg 2, 2628CD, Delft, The Netherlands.
[b]Flexible Optical B.V., Polakweg10-11, 2288GG, Rijswijk, The Netherlands.

ABSTRACT

This proceeding reports early results in the development of a new technique for adaptive optics in confocal microscopy. The term adaptive optics refers to the branch of optics in which an active element in the optical system is used to correct inhomogeneities in the media through which light propagates. In its most classical form, mostly used in astronomical imaging, adaptive optics is achieved through a closed loop in which the actuators of a deformable mirror are driven by a wavefront sensor. This approach is severely limited in fluorescence microscopy, as the use of a wavefront sensor requires the presence of a bright, point like source in the field of view, a condition rarely satisfied in microscopy samples. Previously reported approaches to adaptive optics in fluorescence microscopy are therefore limited to the inclusion of fluorescent microspheres in the sample, to use as bright stars for wavefront sensors, or time consuming sensorless optimization procedures, requiring several seconds of optimization before the acquisition of a single image.

We propose an alternative approach to the problem, implementing sensorless adaptive optics in a Programmable array microscope. A programmable array microscope is a microscope based on a digital micromirror device, in which the single elements of the micromirror act both as point sources and pinholes.

Keywords: Confocal Microscopy, Digital Micromirror Device, Wavefront Optimization, Fluorescence.

1. INTRODUCTION

Adaptive optics are a well proven technology for the correction of aberrations in optical systems[1]. They are extensively used in astronomy and laser optics. Due to the growing interest in imaging in three dimensional samples in fluorescence microscopy, adaptive optics would represent an important evolution of existing fluorescence microscopy techniques[2]. However, several challenges prevent the widespread application of adaptive optics in fluorescence microscopy.

Traditional adaptive optics systems are formed by three components: an active optical element, a wavefront sensor, and a controller. The active element is usually a deformable mirror, the wavefront sensor is usually a Shack Hartmann sensor, while the controller is a computing system which, in a single step, given the aberration detected by the wavefront sensor, calculates the optimal correction to apply on the active element.

The main requirement for a classical adaptive optics system to work, is the presence of a bright, point like source in the field of view of the imaging system, which is necessary for correct operation of a Shack Hartmann sensor[3]. While such requirement is easily satisfied by bright stars in astronomy, the vast majority of biological samples for fluorescence microscopy has low brightness, and no point like objects are usually present. Moreover, imaging in three dimensional samples, where adaptive optics are needed the most, is usually performed through laser scanning microscopy[4], so that, even if a bright point source is present, it would be illuminated for a small fraction of the imaging time, therefore limiting the working frequency of the adaptive optics system.

As a consequence of these limitations, the reported implementations of adaptive optics in scanning fluorescence microscopy are usually divided in two categories: In some cases the traditional wavefront sensor configuration is used[5], with extremely bright synthetic fluorescent microspheres embedded in the sample, while in other applications a wavefront sensorless optimization approach is adopted[6]. While the microspheres approach grants high quality results, the inclusion of such materials in a biological samples is often impossible, and in general not particularly appreciated by the

* p.pozzi@tudelft.nl

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life sciences community. On the other hand, the optimization approach can lead to satisfying results, but has some issues in its application.

Wavefront sensorless optimization is usually performed by estimating a quality metric from the images acquired from the microscope[7]. On a fixed field of view, with a convenient metric, the value of the metric is minimal when the aberrations in the optical system are minimized. Optimization procedures are based on a pseudo-random variation of the degrees of freedom of the system, converging towards an optimal minimum. Such a procedure requires the acquisition of an elevated number of images, since even the fastest reported algorithms require 2N+1 iterations, where N is the number of degrees of freedom of the system[8]. Since laser scanning microscopes have slow image acquisition rates (generally around 1 image per second, for low noise images), and high quality deformable mirrors can have a considerable number of actuators, the optimization procedure can require several minutes to be performed on a single field of view, and every time the field of view is changed, due to the image dependent nature of the metric, the procedure must be entirely repeated.

Due to these limitations, while many approaches have been developed, adaptive optics are still not widely used in general microscopy applications.

2. THEORETICAL CONCEPT

In this paper a newly developed approach to the implementation of adaptive optics in scanning fluorescence microscopy is presented, reporting simulations and preliminary experimental results.

The core of the presented idea is the computation of a optimization metric which can be computed in a time orders of magnitude shorter compared to the scanning image acquisition procedure, so that a sensorless optimization procedure can be performed seamlessly at a high frame rate during the conventional operation of the microscope.

In order to achieve such result, the chosen metric must satisfy some strict requirements:

- Estimation of the metric should be possible continuously during the scanning procedure.

- The metric must be as much as possible independent from the intensity of the fluorescence signal and the location of the scanning pattern.

- Estimation of the metric should be performed without impairing the acquisition of the full image.

In order to satisfy the strict reported conditions, we chose to use, as a metric, the portion of light usually rejected by the pinhole of a confocal system. Supposing to use a pinhole with a reflecting surface, acquisition of images of the pinhole optical plane through a pixelated detector would show the out of focus fluorescence for point like illumination, with a small dark area at the pinhole position. In an aberration free system, all of the fluorescence light emitted in the confocal volume would be focused in the pinhole, however, in the presence of aberrations, some of the confocal fluorescence is rejected by the pinhole.

The spatial extent of the rejected light distribution, described as a metric by the second moment of the pinhole plane image, is expected to be related to the aberration amplitude. In particular, it has previously been proved that such relationship, in the absence of strong out of focus emitters is quadratic, and therefore convex[9]

. Moreover, as long as the signal is detectable, such a metric is independent from the intensity of the fluorescence signal.

The metric described can not, however, be used on a single focus laser scanning, as it is expected from the sample that some areas within the field of view are too dark for proper estimation of the metric, and moreover the aberration is spatially dependent, so that different areas in the sample will require different corrections, and the optimization procedure will not converge.

In order to overcome such problem, this sort of technique requires a microscope which scans in parallel multiple focal points through multiple pinholes, so that it can be assumed that at any moment some bright area of the sample is illuminated, and evaluating the metric on the average of the images of all pinholes the system will converge to an average wavefront correction which will be effective over the full field of view.

In this proceeding numerical simulations of such system, and preliminary results of its experimental application on a modified version of a programmable array microscope[10] (PAM) are reported. A PAM is a digital form of confocal microscope based on the presence of a digital micromirror device (DMD) in an image plane of the system. A DMD is a device consisting on a pixelated optically reflective surface, in which each pixel can be switched between the binary tilt configuration of -12° ("on" position) and +12° ("off" position) with respect to the surface normal.

In a PAM the DMD surface is positioned in an image plane of the system. Excitation light illuminates the DMD surface uniformly from an angle of -24°, so that, in the presence of a sufficient magnification in the microscopy system, each pixel of the DMD can produce a diffraction limited, point like illumination spot in the sample when switched to the "on" position.

When a single pixel of the DMD is switched "on", the fluorescence light generated in the sample, due to Babinet's principle, is focused back on the DMD pixel, which due to its dimensions acts like a pinhole, reflecting only the confocal fluorescence light back towards the light source. Positioning a Dichroic mirror and a fluorescence filter between the light source and the DMD it is possible to use the fluorescence light to image the surface of the DMD on a scientific camera (Imaging camera).

In order to acquire a confocal image, the imaging camera should acquire a long exposure, during which each individual pixel of the DMD is turned "on" once individually, or as part of a non cross-talking subset of pixels. In this way, a optically sectioned image of the microscope focal plane is formed on the camera sensor.

The system is in principle similar to a spinning disk microscope[11], with the important difference of having a reflective pinhole array, instead of a transmissive one. As a consequence, the out of focus light rejected by the pinholes, which in a spinning disk or confocal microscope is just absorbed or scattered, in a PAM is reflected at an angle of +24°, symmetrical to the light path of confocal light.

In the method proposed in this paper, a second scientific camera (optimization camera) is used to detect the out of focus light in an optical scheme symmetrical to that of excitation light. While the imaging camera acquires long exposure images, the optimization camera is refreshed at the highest speed possible, and in particular at a speed faster than the refresh rate of the DMD.

3. SIMULATIONS

In order to characterize the performances of such a system, an accurate numerical simulation of the system has been developed. The simulation was performed through the physical optics simulator "Lightpipes"[12], in its Python version.

Simulation was performed on a single "on" element of the DMD, associated to a 20X, 0.3 N.A. Objective.

In the simulation, the light field was propagated with scaling from the DMD plane for a distance equal to the tube lens focal length (20 cm), then propagated with the Fourier transform method to the objective back aperture. In the objective back aperture a known random aberration, including Zernikes up to the fourth order, and excluding tip, tilt and defocus, was applied to the field. From here the field is propagated with scaling to a total of 100 planes equally spaced within ± 3 ω_z of the unaberrated psf. The sample is modeled as a random three dimensional distribution of point-like objects of equal brightness, with one of them at the location of the center of the unaberrated psf, in the focal plane of the objective.

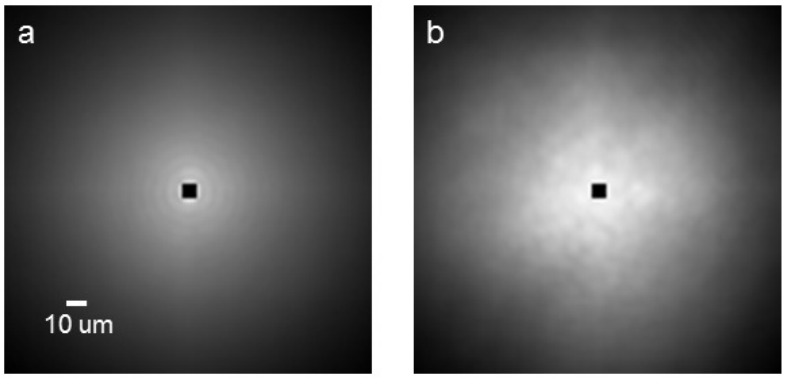

Figure 1: Simulation of light intensity distribution at the DMD plane for direct illumination of a single, under-resolved particle within a three dimensional sample of randomly distributed identical particles. (a) is with unaberrated system, (b) is for an aberration of 10λ rms.

Each simulated point like object is then considered as a light source, with intensity proportional to that of the excitation field at the same location. The field from each object is propagated back to the DMD plane, and the intensity of the fields is summed together to approximate the incoherent nature of the fluorescence emission. A central section of such image, corresponding to the DMD element shape is blacked out, to simulate the output of the optimization camera. Finally, in order to simulate the actual digital output of a camera, a white noise component with amplitude proportional to the square root of the signal was added, together with an offset, and a 16-bit digitalization.

The simulation was repeated, varying the amplitude of the same random aberration, and the metric was calculated as the second moment of the normalized intensity distribution.

Such simulation was run, with the same aberration, for several sample fluorophores distribution, with varying emitters concentrations, in order to prove that the metric only depends on the aberration amplitude, and not on the sample brightness or structure. A summarizing graph is reported in Figure 2. It is possible to observe how the metric only has minor variations depending on the sample characteristic, and a strong correlation to the aberration intensity.

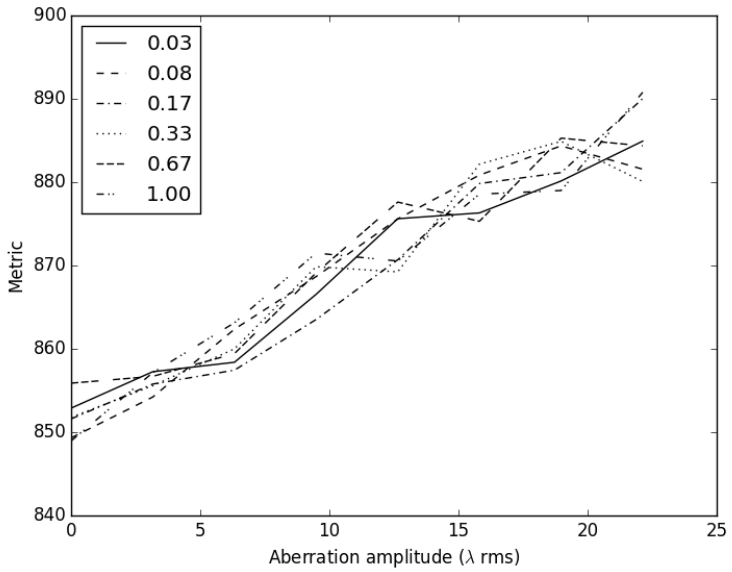

Figure 2: Simulation of metric dependence from aberration amplitude and sample structure. Each plot reports results from the same aberration, with varying amplitude, for different sample structure, with randomly distributed fluorophores at different concentrations. The legend reports the relative concentrations of fluorophores in the plots.

4. EXPERIMENTAL SETUP

A prototype of the described technology is in development. The prototype consists in a custom upright microscope, with excitation source and fluorescence filters optimized for the use with green fluorescent protein. The back aperture of the microscope objective (63X, oil immersion, 1.4 Numerical aperture, Leica, Germany) is conjugated to the aperture of a deformable mirror (DM-69, Alpao, France) through a galileian telescope made by two achromatic doublets (AC-508-150-A, Thorlabs). The deformable mirror is positioned in the back aperture plane of a single achromatic doublet (AC-508-150-A, Thorlabs), with a DMD (Lightcrafter 6500 evaluation module, Texas Instruments, U.S.) in the focal plane.

The surface of the DMD is imaged on two identical cameras (Optimos, QImaging, Canada) at +24° and -24° angles from the DMD surface through two telecentric lenses (TEC-55M, Computar, Japan) in Scheimpflug configuration. On the +24° optical path, a fluorescence filter is present (MF525-39, Thorlabs, U.S.), while on the -24° optical path, a fluorescence filters cube is present, with a dichroic mirror (MD498, Thorlabs, U.S.), and a fluorescence filter (MF525-39, Thorlabs, U.S.).

As an excitation source, a high brightness, 470 nm, 20 W LED is used (PT-121-B-L11-EPF, Luminus, U.S.). An image of the LED surface is formed on the DMD by a condenser lens (ACL3026U-A, Thorlabs, U.S.), and filtered by a bandpass filter (MF469-35, Thorlabs, U.S.). The system is controlled by a custom software developed in Python.

In order to calibrate the deformable mirror, a removable flat mirror is placed in the mid point of the galileian telescope, deviating excitation light towards a short focal achromatic doublet (AC-256-60-A, Thorlabs, U.S.) conjugating the plane of the mirror to the microlens array of a Shack-Hartmann detector (WFS-150-7AR, Thorlabs, U.S.).

5. EXPERIMENTAL RESULTS

Preliminary measurements were performed on a relatively thin and non aberrating autofluorescent sample (Convallaria Slide, Leica, Germany). The system was focused in the sample, with the deformable mirror kept in its best flat configuration. A sample image was acquired as reference, unaberrated result. Subsequently, a random aberration, chosen with zero tip tilt and defocus coefficients in its Zernike expansion, is introduced through the deformable mirror. A live stream of confocal images is then acquired, while a simple gradient descent optimization is performed over the second moment metric of the average of pinhole images on the optimization camera.

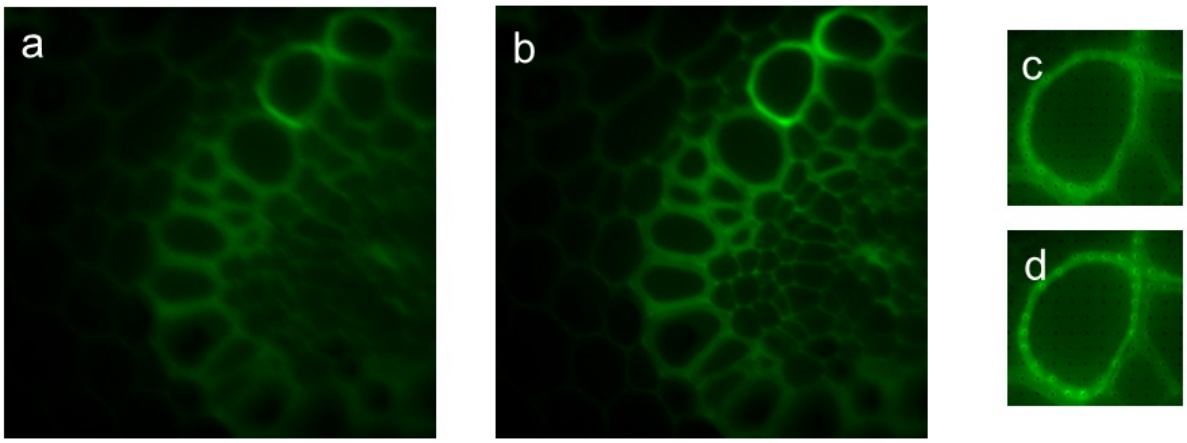

Figure 3: Experimental validation of the method. a) Image acquired with random aberration artificially introduced by the deformable mirror. b) image at the end of the optimization procedure. c) Image acquired by the optimization camera at the beginning of the optimization procedure. d) Image acquired by the optimization camera at the end of the optimization procedure.

The full scan of the field of view is obtained through a sequence of 100 DMD binary patterns, with 5 ms exposure each, so that a complete image is acquired in 0.5 s. The optimization camera is synchronized with the DMD refresh, acquiring images of the pinholes at 200 frames per second. The optimization runs over 66 degrees of freedom, covering the complete range of aberrations achievable through the 69 actuators deformable mirror with the constrains of keeping the tip, tilt and defocus coefficients at zero.

The procedure was repeated 100 times, achieving convergence on an average of ~1000 iterations per trial, corresponding to a total of 10 frames acquired by the imaging camera.

6. CONCLUSIONS

In this proceeding, we reported a new approach to sensorless, optimization based adaptive optics in confocal microscopy. This new method is implemented in a programmable array microscope, and is based on the optimization of signal intensity independent metrics computed on the images obtained through out of focus light conventionally rejected by the microscope's confocal pinholes. The great advantage in the use of such technique lies in the possibility of computing the metric at frequencies much higher than the frame rate of the microscope itself, therefore allowing faster convergence of the optimization.

Simulations of the setup operation principles confirmed the convexity of the function relating the values of the metric with the amplitude of the aberration, and its independence from the sample structure.

Preliminary tests performed on an experimental setup showed good convergence times, especially considering the very simple optimization algorithm employed. As a comparison, the algorithm employed, with N=66 degrees of freedom, required an average of 1000 iterations to converge, while high performance algorithms have proven capable to converge, on similar systems, in 2N+1=133 iterations. However, due to the high frequency of metric estimation in the system, our setup was capable to reach optimal performances in a total of 10 image frames, while a standard, image based optimization technique, even with state of the art algorithms, would have required 133 frames. The increase in convergence speed achieved was therefore ~13 fold.

In the immediate future we will implement in the system a more efficient optimization algorithm, in order to achieve true real time correction, and test the system on sample-induced aberrations in three-dimensional samples.

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