Development and testing of an AO-structured illumination microscope

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ABSTRACT

The design of an Adaptive Optics (AO) Structured Illumination (SI) microscope is presented. Two key technologies are combined to provide effective super-resolution at significant depths in tissue. AO is used to measure and compensate for optical aberrations in both the system and the tissue by measuring the optical path differences in the wavefront. Uncorrected, these aberrations significantly reduce imaging resolution, particularly as we view deeper into tissue. SI allows us to reconstruct an image with resolution beyond the Rayleigh limit of the optics by aliasing high spatial frequencies, outside the limit of the optics, to lower frequencies within the system pass band. The aliasing is accomplished by spatially modulating the illumination at a frequency near the cutoff frequency of the system. These aliased frequencies are superimposed on the lower spatial frequencies of the object in our image. Using multiple images and an inverse algorithm, we separate the aliased and normal frequencies, restore them to their original frequency positions, and recreate the original spectrum of the object. This allows us to recreate a super-resolution image of the object. A problem arises with thick aberrating tissue. Tissue aberrations, including sphere, increase with depth into the tissue and reduce the high spatial frequency response of a system. This degrades the ability of SI to reconstruct at super-resolution and limits its use to relatively shallow depths. However, adding AO to the system compensates for these aberrations allowing SI to work at maximum efficiency even deep within aberrating tissue.

Keywords: Adaptive optics, Microscopy, Super-resolution, Structured Illumination

1. INTRODUCTION

While super-resolution imaging has revolutionized optical microscopy by overcoming the diffraction limit in far field imaging, it has yet to transform dynamic live, in-vivo, imaging through thick tissue, which is of broad interest to the biological research community.[1] Super-resolution imaging through thick tissue will first require achieving diffraction limited imaging, which can be impacted by aberrations in the sample and the optical system. Our design for an Adaptive Optical Structured Illumination Microscope (AO-SIM) is made to be capable of live (1 Hz frame rate), in-vivo dynamic imaging in thick specimens (>90 μm) at half the diffraction limited resolution (~100 nm). Our approach is also applicable to other forms of structured and super-resolution illumination imaging including Stimulated Emission Depletion (STED) microscopy, Photo-Activated Localization Microscopy (PALM), Stochastic Optical Reconstruction microscopy (STORM), 3D-SIM and Selective Plane Imaging Microscopy (SPIM).

The requirements for performing high-resolution imaging for deep tissue are to accurately measure and compensate the wavefront error introduced by the tissue. This is particularly challenging for live biological tissue as the sample's composition is continuously changing, thus changing the wavefront error introduced by the tissue. The key element is to compensate the wavefront at a faster rate than the changes going on inside the sample. To accomplish this task the microscope will utilize a fast and direct wavefront measurement component known as the Shack-Hartmann Wavefront Sensor (SHWS).[2] The measured wavefront error will then be corrected by a dual correction system, where the aberrations introduced by the tissue will be corrected by two Deformable Mirrors (DMs): A "woofer" DM that will correct the high-magnitude but low-order (i.e. low spatial frequency) aberrations and a "tweeter" that will correct the low-magnitude, high-order aberrations.[3] This woofer-tweeter configuration will allow us to increase the imaging depth capability to 100μm. This adaptive optics system will allow for full utilization of the super-resolution capabilities of the structured illumination microscope for live imaging by precisely measuring and correcting the wavefront error in the low and high-order aberrations at a greater depth (>90 μm) and at a higher speed (0.6 sec AO correction time) than is currently possible, as described below in the comparison of performance criteria to currently available technologies.

The system design is oriented towards live in-vivo imaging through thick tissues. One important science case that exemplifies this requirement is the study of chromosome structure and function during meiosis. One major barrier to imaging chromosome structure during meiosis the is the fact that the early stages of this process will typically occur towards the middle of the embryo, and thus imaging these early stages of meiosis requires focusing through the embryo material, which causes aberrations. For effective imaging of this process, the system requires the ability to perform dynamic in vivo imaging through thick tissue (10-100 μm) with 1 Hz frame rates and 100 nm resolution. This level of super-resolution has also enabled imaging of the chromosome structure in *C. elegans* for limited depth below the coverslip (16 μm), where the depth was limited by the aberrations that we propose to correct.[4] The development of an AO system to be paired with this microscope will allow these types of studies to be carried out deeper in the target cell, and thus earlier on in the meiotic process.

2. THEORY AND BACKGROUND

2.1 Structured Illumination Theory

Spatial information of an object is limited by the OTF of the optical system used to view it. The cutoff frequency of the system is determined by its optical properties and is generally given as $f_o = 2 * NA / \lambda$. Spatial frequencies above this value are not passed through the system and hence the details of the object that involved those frequencies are not present in the image, limiting detail.

Structured illumination microscopy can increase the resolution of a wide-field microscope, by bringing spatial frequencies that are above the cutoff frequency down into the pass band of the lens. This is accomplished by utilizing a spatially modulated source, with a frequency close to the cutoff frequency, to illuminate the object in focus. The spatial modulation aliases the frequencies above the modulation frequency into the pass band of the system, overlapping the frequencies that are naturally there. Using Fourier domain processing, we can algorithmically separate the normal frequencies from the aliased and overlaid frequencies using multiple images, with multiple parameters of the modulation. We then move the aliased frequencies back to their original position and reconstruct an image of the object with the higher frequency detail than the system alone could image, increasing its resolution.

Structured illumination can also provide axial sectioning. The OTF of a system with defocus has much lower gain at the high spatial frequencies than a system with no defocus. Objects that are out of focus will therefore have less high spatial frequency information than those in focus. Additionally the modulation illumination will be weaker as well. Since the reconstruction algorithm relies on the structure of the illumination to alias the higher spatial frequencies and bring them into the pass band of the system. It effectively selects the high frequencies of those objects that are in the focal plane to reconstruct and thus performs an axial sectioning of those images.

This method can achieve the highest resolution when the optical system also has the highest resolution. However, if there are aberrations in the light path caused by the sample or the system, the structure of the illumination will be degraded. This reduces both the amount of detail in the structured illumination that can be projected into the object and the detail that can be passed through it system. These kinds of aberrations increase as we try to view deeper into the tissue. If the aberrations in the optical path are too large, the illumination pattern imaged onto the object at the focal plane degrades and there is less ability to reject the out of focus light or alias higher spatial frequencies. Consequently, our ability to reconstruct the supper resolution image also degrades. [5][6]

In fluorescent microscopy for biological imaging, structured illumination has sparked a new life by providing an inexpensive, high-resolution technique for acquiring wide-field images. But, deep tissue imaging is still a major limitation for structured illumination microscopy. The tissue in the optical path introduces aberrations that degrade the imaging capabilities. This problem becomes more pronounced as the depth of the object of interest increases since more aberrations are introduced in the optical path, particularly sphere, which similarly to defocus reduces the gain at high spatial frequencies. This problem can be overcome by correcting the aberrations in the optical path. Adaptive optics has proven to be a very useful tool in overcoming this limitation, not just in wide-field microscopy but also in other high-resolution imaging techniques like confocal and multi-photon microscopy[7].

$$I_{ill}(x,y) = H(x,y) * I_{sfp}(x,y) \quad (1)$$

Where $I_{ill}(x,y)$ is the image of the structured illumination at the object plane, $H(x,y)$ is the intensity point spread function of the imaging system, $I_{sfp}(x,y)$ is the intensity of the single frequency illumination pattern, and * is the convolution functioin:

$$I_{sfp}(x,y) = 1 + m\cos(k_g x + \theta) \quad (2)$$

Where m is the modulation depth, k_g is the illumination pattern normalized spatial frequency, and θ is an arbitrary phase for the grating. In the Fourier domain the image produced at the object plane is given by:

$$F_{ill}(f_x, f_y) = \hat{H}(f_x, f_y) F_{sfp}(f_x, f_y) \quad (3)$$

Where $F_{ill}(f_x,f_y)$ and $F_{sfp}(f_x,f_y)$ are the Fourier transforms of the structured illumination and the intensity pattern respectively, and $\hat{H}$ (f_x,f_y) is the optical transfer function of the imaging system.

To produce an optical sectioned image of the object of interest, three different images are collected with three different phases (I_1, I_2, I_3 when θ =0, 2π/2, 4π/3). Each image contains information pertaining to the object and structure illumination as follows[12]:

$$I_n(x,y) = I_{ill}(x,y) O(x,y) * H(x,y) \quad (4)$$

Where $O(x,y)$ is the object of interest, and $I_n(x,y)$ is the structured illumination image (n=1,2,3) obtained for each phase (θ =0, 2π/2, 4π/3). The sectioned image can then be mathematically produced by using the following equation on each pixel of the previously collected images I_1, I_2, I_3: d

$$I_s = \sqrt{\frac{(I_1 - I_2)^2 + (I_1 - I_3)^2 + (I_2 - I_3)^2}{2}} \quad (5)$$

Where I_s is the structured illumination sectioned image.[8]

2.2 Resulting need for adaptive optics

As can be evidenced by equations 1-4**,** the success of the SIM reconstruction are heavily influenced by the PSF of the optical system and sample. While the optical system itself can be designed to be diffraction limited, the wavefront distortions introduced by the imaging through deep tissue samples are dynamic and depend on the characteristics of the tissue used, imaging depth and NA of the objective. In general, the deeper the imaging depth, the stronger the optical aberrations and the more the PSF is spread out and distorted. Thus in order to successfully reconstruct a super-resolution image beneath the sample surface, we need to use Adaptive Optics to correct for the aberrations and shrink our PSF back to a compact, diffraction-limited point. In particular, we expect that our deep tissue PSF will be distorted by a very strong amount of spherical aberration, which our selection of AO hardware, described below, has been optimized to remove.

3. HARDWARE SETUP

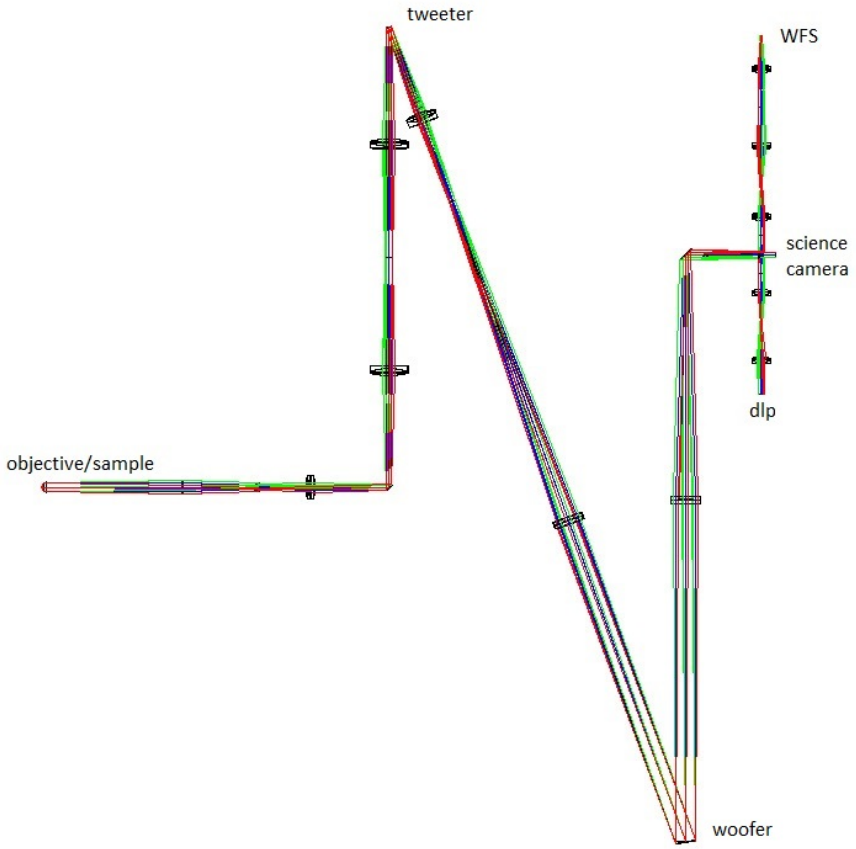

Figure 1. Optical layout of our AOSIM test bench

Figure 1 shows the optical set-up for the proposed Adaptive Optical Structured Illumination Microscope. To simplify the design and prove the adaptability of the AO-SIM, the set-up will use an Olympus IX 71 inverted microscope frame. Our sample at the current stage of testing consists of GFP microdots embedded in ~100 microns of agar on a standard microscope slide. We will adapt this to fully test the AO system capabilities by putting the microdots underneath Drosophila embryos, which will introduce significant aberrations and scattering on the way to and from the microdots.

3.1 Illumination and DLP leg

Our illumination leg consists of a 60mW 488nm cyan laser. This wavelength was chosen due to its suitability for exciting the GFP microdots. The laser will travel through a spatial de-correlator that will greatly reduce the coherence of the light. The resulting beam then hits a (608 x 684 PIXEL) TI DLP, which we use to create a binary structured illumination image in our laser light. While the ideal illumination pattern is a continuous sine wave, the extra high frequency components of our binary structure pattern is filtered by the system optics so that only the primary frequency is left.

3.2 Wavefront Sensing and Correction

The wavefront correction leg of our optical system consists of two deformable mirrors, a woofer and a tweeter, both positioned conjugate to each the system pupil. The woofer is a 52 actuator Imagine Optics MirAO 52e magnetic actuator mirror. It was selected due to its high stroke (40 microns) and thus its suitability to correct low order aberrations, such as the strong spherical aberration typically produced by thick biological samples. The tweeter is a 140 element Boston

Micromachines MEMS DM. It was chosen for its high actuator count, which we will use to correct the residual high order aberrations left after removing the low order aberrations with the woofer.

To take full advantage of the adaptive optics system compensation the proposed optical design of the system corrects both the structured illumination light on its way to the object plane and the light that it gathers from the object plane for imaging.

The wavefront data used for controlling this mirror will come from a Shack-Hartmann wavefront sensor, consisting of a 12x12 lenslet array projected onto a Princeton Instruments PhotonMAX camera. This approach was chosen over an iterative image-optimization technique due to its faster speed, allowing our system to image in real time.

4. DATA COLLECTION AND RECONSTRUCTION

4.1 Noise removal

The effectiveness of the reconstruction is also heavily affected by the image noise. As such, care must be taken to remove background noise without removing any real data. An effective first step towards this to select an integration time and gain that puts the desired signal near saturation without actually saturating the camera. This ensures that the image noise is far less intense than our signal, and can thus be separated out more easily. Next we obtain a background noise image, in which a series of images are taken with no illumination at the desired gain and integration time, then averaged together. This background is then subtracted from any images taken afterwards. The resulting image has a very strong signal and low residual background noise, making it easy to remove the remaining noise via thresholding without removing any actual image data.

4.2 Algorithm operation and iteration

The reconstruction process is being performed in Matlab after data capture. In addition to performing the matrix operations required for reconstruction, the program serves to determine appropriate initial conditions and parameters for each new data set. Some of these parameters are variables in the reconstruction equations above such as the SIM shift frequency and initial phases, but there are also other parameters that are necessary in order to apply the algorithm to pictures obtained in real optical systems, such as 'skew' (the unintentional offset angle of the illumination pattern) and the cutoff frequency of the filters used to remove high frequency noise from our images before and after reconstruction. Theoretically, in an implementation with a fully fixed/static optical system and illumination pattern, these parameters will only need to be determined once, but in any real practical implementation, some small shifting will likely occur over time, and thus this calibration can likely be performed once per imaging session rather than once per image set as in our current system.

We have found that a good procedure is to start by iterating over the skew to select the angle that reduces image artifacts. Next, the SIM frequency is iterated over, although a very good initial estimate can be given by observing the fft of the pattern sent to a reflective target rather than the fluorescent dots. The best SIM frequency will show the most compact dots upon reconstruction. We also must iterate over both the x and y initial phases, and select the settings that lead to the least shift in the resulting image. Some inconsistency over the shift amount across the reconstructed image can point out if more tuning is required to select the appropriate SIM frequency. The final parameter that is iterated over is the cutoff of the filter used on each individual image before reconstruction, and the filter used on the disentangled/shifted images. Ideally, this should match the cutoff of our system optics pretty well. As such, we can get a pretty good estimate of this by created a series of points on an image, and increasing the cutoff of the filter applied to it until the resulting dot size is similar to that of the smallest point features being viewed by the microscope. Through iteration we can fine tune this number, as too low a cutoff will result in image artifacts, and two high a cutoff will result in image noise and artifacts.

5. RECONSTRUCTION RESULTS

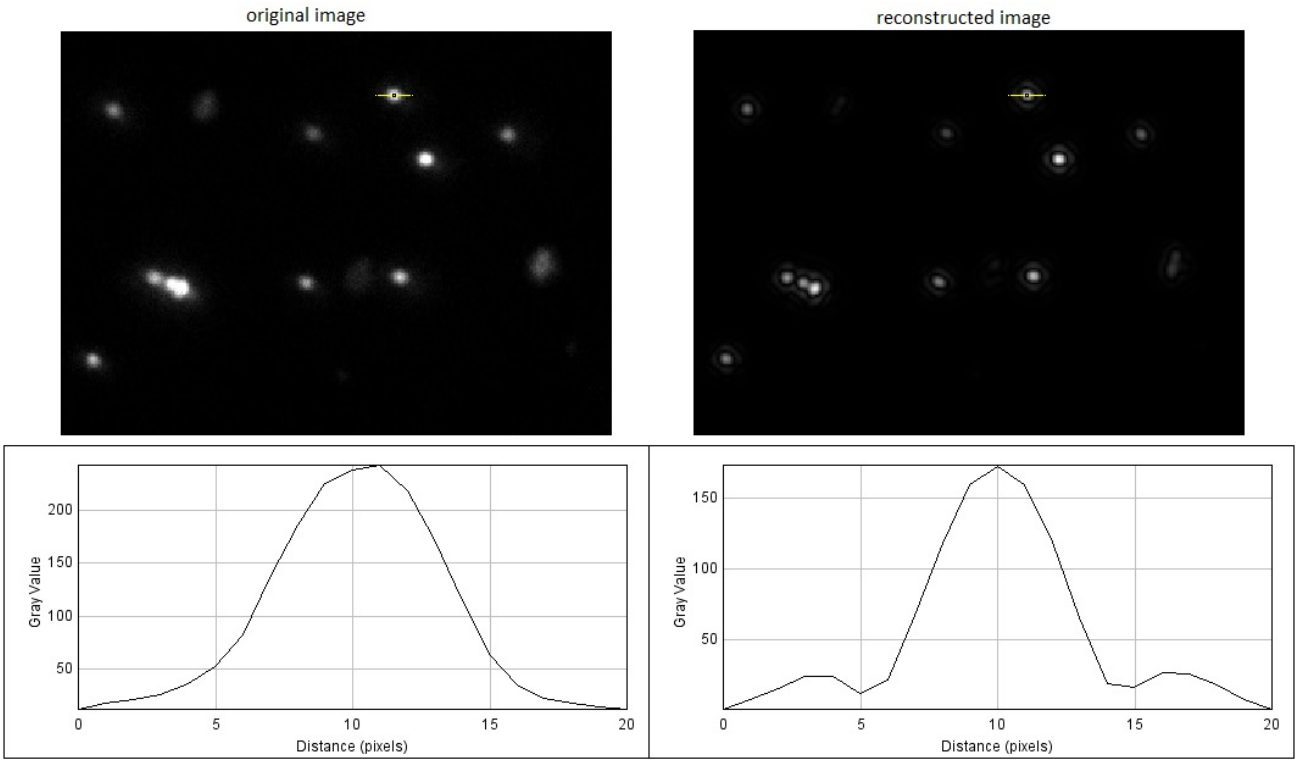

Figure 2. Comparison of original and reconstructed images

An example of the reconstruction algorithm after all parameters have been selected can be seen in figure 2. A few effects of the reconstruction can be seen. First, all in focus dots have been decreased in size and have more brightness concentrated in the core. This can be seen especially well in the dots that were close enough to not display clear separation on the original image. In the reconstructed image, these dots are smaller and show a clear decrease in intensity between them, distinguishing them as separate dots, as can be seen by the line profile. Another prominent effect of the reconstruction is the rejection of out of focus signal. On the original image, these out of focus signals are the dots that are much wider and dimmer. On the reconstruction, these out of focus dots either decrease in intensity or disappear altogether.

The rejection of the out of focus dots also exemplifies the need for AO in order to effectively reconstruct through aberrating tissue. Other distortions in the patterned illumination's wavefront, such as sphere and coma, will similarly distort the image PSF, causing some of the aberrated signal to be rejected by the algorithm.

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